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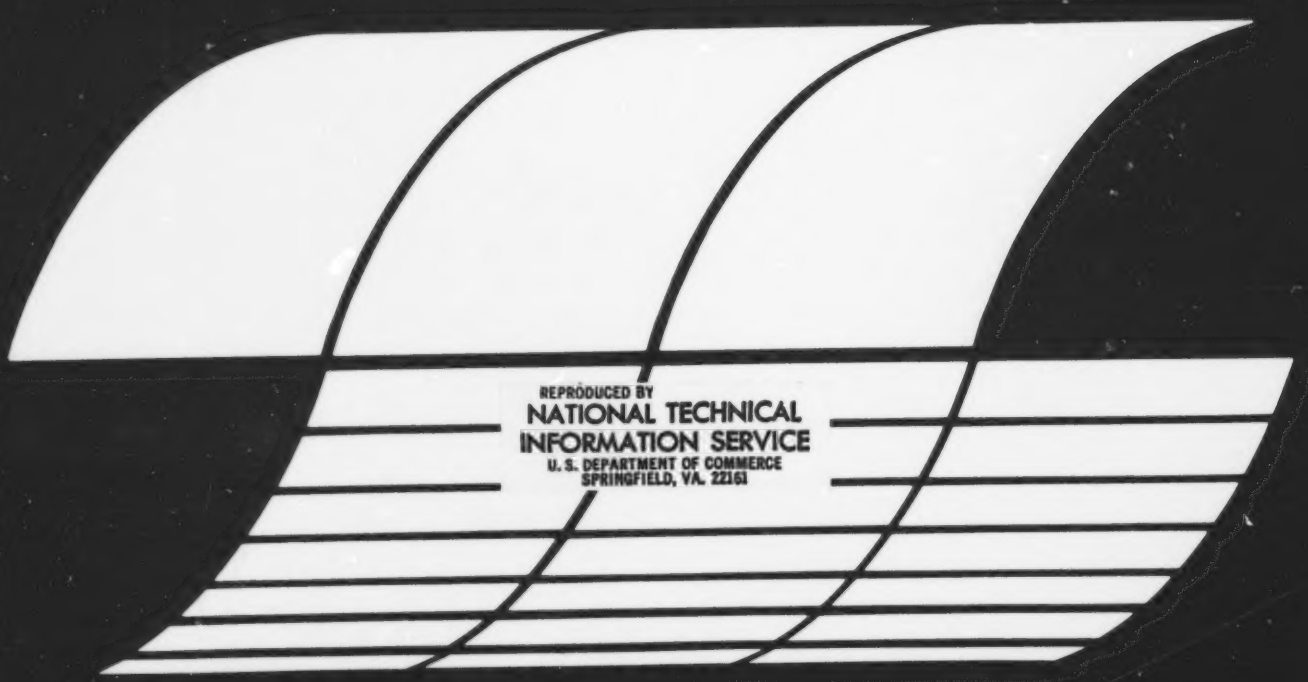

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The responses to Prudhoe Bay oil of the microbial populations present in water column, beach and sediment samples representative of the diverse marine shoreline environments found in the northern Puget Sound and Juan de Fuca areas were investigated under laboratory conditions. All sites studied yielded psychrotrophic bacterial populations which, in the presence of added nitrogen and phosphorus, were able to bring about changes in the n-alkane components of Prudhoe Bay oil. Under laboratory conditions, apparently one-third of the weight of oil was lost by weathering, one-third lost by mineralization due to microbial activity and one-third left as a residue. No changes in Prudhoe Bay oil were observed with nitrogen and phosphorus being absent. Increasing the temperature of incubation resulted in a faster rate of disappearance of n-alkanes present in this oil. Samples from the water column environment showed the greatest seasonal variation in ability to modify Prudhoe Bay oil. Beach samples were quite constant in their ability to yield oil-degrading bacterial populations.

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MICROBIAL DEGRADATION OF PETROLEUM HYDROCARBONS

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FOREWORD

Substantially increased petroleum tanker traffic, pipeline transport and refining operations are anticipated in the region of Northern Puget Sound and Strait of Juan de Fuca with the Alaskan pipeline in operation. To assess the potential future environmental impact arising from these activities the Puget Sound Energy-Related Project, contracted the University of Alberta to undertake a study on the "Microbial Degradation of Petroleum Hydrocarbons" in Northern Puget Sound and Strait of Juan de Fuca. This study was supported by U.S. Environmental Protection Agency "pass-through" funds administered by the NOAA Marine Ecosystem Analysis Program. This report presents the results of the study.

ABSTRACT

The responses to Prudhoe Bay oil of the microbial populations present in water column, beach and sediment samples representative of the diverse marine shoreline environments found in the northern Puget Sound and Juan de Fuca areas were investigated under laboratory conditions. The presence and activity of oil-degrading microbial populations were monitored by determining changes in the n-alkane components of the saturate fraction of oil by gas chromatography. Samples were enriched in a nitrogen, phosphorus-deficient synthetic medium with Prudhoe Bay oil as sole carbon source. Under these conditions no degradation of Prudhoe Bay oil was observed after either 28 days incubation at 8°C (typical ambient temperature conditions for water in this area) or 14 days incubation at 30°C. However when nitrogen and phosphorus were added to the medium, all sites yielded bacterial populations capable of utilizing the n-alkanes of Prudhoe Bay oil. The rate of utilization was faster at 30°C than at 8°C. Under laboratory conditions approximately one-third of the total weight of Prudhoe Bay oil was lost by weathering (i.e. physical-chemical processes), one-third by mineralization due to microbial activity and one-third left as a residue. No yeasts or fungi were isolated using these procedures.

Seasonal variations in oil-degrading capabilities were compared by calculating a "degradative capacity index" (D.C.I.). The index describes the status of the n-alkane profile of the saturate fraction of degraded, partially degraded and undegraded Prudhoe Bay oil. If all samples studied contained microorganisms which completely utilized the n-alkanes and isoprenoids present in an oil a D.C.I. value of four would be obtained. If none of the samples contained such microorganisms the D.C.I. would be zero. Water column samples showed the greatest seasonal variation in their D.C.I. values, being the lowest in the winter (i.e. January). Beach samples were quite constant in their ability to yield bacteria capable of utilizing saturate components.

Inter-tidal sediment samples showed some seasonal variability with regards to the oil-degradative capacity.

Indigenous bacterial populations from sediment, beach and water column samples primarily consisted of Gram-negative bacteria. Oil-degrading populations contained relatively high proportions of members of the Gram-negative genera Pseudomonas and Flavobacterium with varying proportions of Gram-positive coryneforms. There was no apparent relationship between sample type or site and the generic composition of bacterial mixtures capable of degrading Prudhoe Bay oil.

Initial studies on the rate of oil degradation using a radioisotope technique also are discussed.

Sediment samples were stored up to six weeks and water column samples for twelve days without loss of ability to degrade Prudhoe Bay oil.

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SECTION 1

INTRODUCTION

The Straits of Juan de Fuca/northern Puget Sound area have experienced a continuous increase in oil-tanker and refinery activities over the past decades. The hydrocarbon demands of the Pacific Northwest and the rest of the United States coupled with the completion of the Alyeska Pipeline indicate that the scope of oil-related activities will continue to increase in the future. As a result of this accelerated activity there is an increased risk of an oil spill taking place.

The ability of bacteria, yeast and fungi (i.e. microorganisms) to utilize hydrocarbons as sources of energy and cell carbon is a characteristic which is widespread in microorganisms but seldom found in other forms of life (1). This capability together with the ubiquitous distribution of microorganisms in aquatic and terrestrial environments provides a biological system which contributes to the natural clean-up of oil spilled in the environment. Information on the distribution and factors affecting the activities of oil-degrading microorganisms in the marine environment has been the subject of recent reviews (2-4). However since no data is available for the Strait of Juan de Fuca/northern Puget Sound area the primary objective of this study was to investigate the distribution and factors affecting the activity of oil-utilizing microorganisms under the environmental conditions prevalent in this area. Studies were designed to obtain information on the effect of geographic variation, seasonal variation and proximity to oil sources on the activity, distribution and types of oil-degrading microbial flora found in this area.

Microorganisms are intimately involved in the maintenance of the biological food chain. An understanding of factors, e.g. oil, affecting the presence and activities of microorganisms is essential to the understanding and management of natural ecosystems. Therefore a secondary object of this study was to investigate the effect of oil on the indigenous bacterial flora of water, sediments and beaches representative of the diverse types of marine environment found in this area.

Studies on the enumeration of hydrocarbon-utilizing microorganisms have been concerned with their incidence as a fraction of the normal heterotrophic population (5). The problems encountered in such enumeration studies have been investigated by Walker and Colwell (6). Such experimentation has shown that oil-or hydrocarbon-polluted areas have higher levels of hydrocarbon-utilizing microorganisms than adjacent unpolluted areas. In general, these studies have been carried out using a pure hydrocarbon, such as an n-alkane

e.g. hexadecane as carbon source, the hypothesis being that the ability to grow on a pure n-alkane is related to the ability to grow on a whole crude oil. However data are available which show that the ability to grow on pure hydrocarbons does not necessarily correlate well with an ability to grow on crude oil even though the n-alkane component is present in that oil. For example, fungi are noted for their ability to grow on n-alkanes (7), although few studies are published on their ability to grow on oils. Davies and Westlake (unpublished results) determined the ability of such fungal isolates including Cladosporium resinae, and new fungal isolates obtained by a crude oil enrichment technique, to utilize crude oils. A very poor correlation was observed between utilization of an n-alkane (i.e. tetradecane) and the ability to grow on crude oils containing n-alkanes. Information (8-10) also is in the literature showing that the chemical composition of crude oil can influence the microbial and thus the chemical responses observed. In order the consider the interaction of different compounds present in oil, studies on the distribution of oil-degrading microorganisms should be carried out using a whole crude oil typical of those which might be spilled in the area.

The measurement of the microbial growth by monitoring the increase in cell mass or numbers when a complex material such as oil is used as substrate does not yield information as to what compound(s) is(are) supporting growth. Growth can be monitored indirectly by following changes in the concentration of the substrate(s) present (11). Of the four major components of crude oil i.e. asphaltenes, saturates, aromatics and the polar N.S.O. (nitrogen, sulfur, oxygen) - containing molecules only the n-alkanes (and to a lesser extent the isoprenoids) of the saturate fraction (8) and the mono-, di- and tri-ring compounds of the aromatic fraction (12, 24) are readily degraded by microorganisms. Changes in the content of the n-alkanes and the isoprenoids can be readily detected by gas chromatography (G.C.). While the aromatic compounds can be resolved by G.C. their resolution depends on a more rigorous pre-treatment of the oil than is required for monitoring changes in the n-alkanes and isoprenoids. The approach used in these studies to assess the distribution and factors affecting the activity of oil-degrading microorganisms was based on an enrichment technique utilizing crude oil (Prudhoe Bay) as sole carbon and energy source. Changes in the n-alkane and isoprenoid components of recovered oil were monitored by G.C. and used as an indicator of oil-degrading microbial activity.

Weathering, i.e. the loss of volatile components by physical-chemical processes, and mineralization, i.e. the conversion of organic carbon to CO_2, are natural processes by which oil may be removed from the environment. While the volatile components contain the most toxic components of oil, they also contain some of the more biodegradable compounds, and thus their loss will affect the amount and types of microorganisms which will grow on oil. The weathering also can result in the formation of structures, e.g. tar balls, which are resistant to microbial attack. Thus the interaction between these processes can affect the rate at which oil is removed from the environment by natural processes. The relationship between these two processes and loss of Prudhoe Bay oil in water column and sediment enrichments was determined under laboratory conditions using a gravimetric procedure. Intial experiments also were carried out on the application of a technique using the rate of $^{14}CO_2$ liberation from ^{14}C-hexadecane-"spiked" Prudhoe Bay oil as an

indicator of the rate of n-alkane removal.

The activity of oil-utilizing microorganisms has been shown to be controlled by temperature (13-15) and by the nutrient status, especially nitrogen and phosphorus content (15-18, 23), of the environment. Thus the effects of these parameters on the activity of microbial oil-degrading populations also were investigated in the Strait of Juan de Fuca/northern Puget Sound area.

SECTION 2

CONCLUSIONS

The results of the survey for the presence of oil-degrading microorganisms in water columns, beach and sediment samples representative of the marine environments found in the northern Puget Sound, San Juan Islands and the southern shore of the Strait of Juan de Fuca indicate their ubiquitous presence in these areas. Microbial psychrotrophic (cold-tolerant) and psychrophilic (cold-requiring) populations will predominate as the temperatures of these marine environments are in the range of 5°C to 20°C.

The enrichment procedures used resulted in the isolation of mixed populations of psychrotolerant, Prudhoe Bay oil-degrading bacteria. No yeasts or fungi were observed under these experimental conditions. The oil-degrading bacterial populations obtained primarily consisted of members of the Gram-negative genera Pseudomonas or Flavobacterium and the Gram-positive coryneform groups. The proportion in which members of these groups occurred varied but was not related to a particular sample type, sample site or enrichment condition e.g. temperature. However in one sample, sediment from False Bay, San Juan Island, members of the Alcaligenes genus comprised the majority of the Gram-negative bacteria found on the oil-degrading population. All of these taxonomic groups have been reported to contain oil- and/or hydrocarbon-degrading organisms. Members of these groups also were present in unenriched indigenous bacterial populations.

Oil-degrading activity of these bacterial populations, as measured by determining the degradation patterns of the n-alkane and isoprenoid profiles of recovered oil, increased with increasing temperature. Activity however was observed only when sample materials were incubated with a nutrient supplement containing nitrogen and phosphorus. The level of nitrogen present was more critical in determining the activity observed than was the level of phosphorus. If an oil spill occurs in this area, then the residual oil left after physical-mechanical processes have been used to recover oil would only be rapidly degraded if nitrogen and phosphorus were provided, theoretically in an oleophilic form. Under these conditions the ambient temperature, not the nutritional status of the environment - would become the rate-limiting parameter. Similarly previous laboratory studies reported that nitrogen and phosphorus levels controlled the rate of oil degradation on northern soils. The application of fertilizer containing nitrogen and phosphorus to actual spills in that area accelerated the rate of oil degradation and the rate of revegetation of such sites.

Under laboratory conditions used, approximately 1/3 of the weight of Prudhoe Bay oil was lost by weathering, 1/3 lost by mineralization leaving a residue of 1/3 of the original weight of oil. Similar values were obtained at both 8°C and 20°C.

The bacterial population in "pristine" areas such as Rosario Beach and West Beach (Deception Pass Park) which contain low levels of organic matter and are relatively free of oil-industrial activities, is more sensitive to added oil than that present in organic matter-rich areas like March Pt. Rd., E. Fidalgo Rd. and the refinery site (Cherry Point) which are also near oil-industrial activities. The growth responses of oil-degrading populations from these two environments, while of a different order of magnitude, are similar. Initial studies on the rate of n-alkane utilization indicate that water column bacterial populations from sites near oil-industrial activities have faster degradation rates than observed in water column samples from "pristine" areas. Sediment samples from these two types of environment however gave similar rates of n-alkane degradation.

Sediment samples also can be stored for up to 6 weeks without loss of oil-degrading capabilities. Water column samples have been stored for twelve days at 8°C without loss of oil-degrading capabilities although significant increases in viable populations occurred.

SECTION 3

RECOMMENDATIONS

The results of this initial survey indicate that the Pt. Partridge Park and the E. Fidalgo sites represent the extremes of the marine environment studied regarding bacterial oil-degrading capability. These two sites should be the subject of an intensive investigation. The nature of the indigenous bacterial populations present, the rate of oil-degradation and the effect of oil on the indigenous bacterial populations should be investigated. Samples from these sites should be limited to water column and beach material since sediment samples (as defined in this study) represent a continuously changing transition zone between water column and beach materials. All samples should be analyzed for nitrogen, phosphorus, organic carbon, oils and greases and oil-degrading capability. Representative samples should also be subjected to enrichment procedures which would select for oil-degrading yeast and fungi. The effect of nutritional conditions - the nitrogen-phosphorus levels used and presence of iron or yeast extract - should also be investigated on samples representative of these two areas. Sampling times must be scheduled so as to confirm the seasonal trend noted for water column samples. These studies should be complemented by parallel investigations with Port Angeles area as a site representative of industrial activity (particularly areas of known hydrocarbon content) and Freshwater Bay as being representative of a "pristine" control. Studies to date have centered on the use of Prudhoe Bay oil as carbon and energy source. As the refineries at Anacortes and Cherry Point are receiving other oil and as it has been clearly shown that oils differ in their biodegradability characteristics (and their toxicity), the study of oil-degrading activity at the above sites should also be measured against oils which are presently being transported in this area.

As many beaches in this area consist of rocks and cobble stones the ability of microbial populations in these areas to respond to oil should be investigated. Similarly a study of the ability of plant material e.g. kelp to harbor oil-degrading microorganisms and the effect of oil on their epiphytic microbial flora should be undertaken.

The presence and effect of oil on sub-surface microbial populations should be studied. In particular a comparison should be undertaken of sub-surface water samples from the Strait of Juan de Fuca (which should have a low sediment content) with high sediment-containing samples from waters affected by the Fraser or Skagit River drainage systems. Similarly, subtidal sediments from hydrocarbon-polluted areas and pristine areas should be investigated. The degradation rate of oil in sediment material low in oxygen should be studied, as the long term effects of oil pollution will be

expressed in this environment.

Consideration should be given to the evaluation of the effect of generic composition of oil-degrading populations on the rate and chemical changes brought about in oil. In particular, there is a need for the development and acceptance of a taxonomic classification system which if adopted would make possible meaningful comparisons between results obtained in different laboratories.

Comparisons of rates of oil-degradation should be expressed on a surface basis as well as on a per gram and/or ml basis as particle contents from different sites will have different surface areas depending on sediment composition. All experiments should be carried out under psychrophilic conditions i.e. around 4°C to 8°C as this represents the rate-limiting minimum for this parameter in this area.

An integrated "in situ" study involving chemists, microbiologists and biologists at a selected site to determine the environmental effect of oil spills on the total ecosystem should be undertaken. The data provided not only would outline the effect of an oil spill but would also possibly permit the development of procedures to minimize oil's effect on the marine environment in this area.

SECTION 4

MATERIALS AND METHODS

Sample Sites

The sites used in this study are shown in Figure 1, and their locations (latitude and longitude) described in Table 1. The environmental codes characterizing each sample site are available in NOAA's computerized data bank. Sites were chosen to represent the diverse environmental situations encountered in this study area and on the basis of their accessibility. Whenever tide and terrain permitted, three samples - a water column, a sediment and a beach sample - were obtained at each site. All materials were transported in cooler chests containing sufficient ice packs to keep the temperature at 10°C ± 5°C.

Water samples were obtained at approximately 30 to 45 cm depth using a wide mouth 4 litre plastic bottle. Microbial, physical and chemical examinations were carried out on a sample which was obtained after rinsing the bottle three times with the water to be examined. Large volumes of water for laboratory use were taken and transported in sterile 4 litre bottles.

In this survey sediment samples refer to the sand which was saturated by tidal action (mid to low intertidal) and beach samples refer to sand above the detritis (upper intertidal) left by spring tidal action. Two hundred to five hundred grams of surface composite (1 cm depth) sediment or beach materials from approximately a 0.5 square meter area were transferred using sterile tongue depressors to sterile 250 ml centrifuge bottles. Beach and sediment samples were stored at -20°C unless otherwise indicated.

Samples were obtained from sites as indicated in this report in April, July, September, November, 1977 and January, 1978.

Microbiological Survey and Activity Studies

The chemical composition of all microbiological media used in this study is reported in Appendix A. An enrichment technique using a mineral medium with a neutral pH and Prudhoe Bay oil as sole carbon source was used to screen for the presence of oil-degrading microorganisms. The effect of nutrition (i.e. nitrogen and phosphorus levels) on oil-degrading activities of such populations was investigated using this technique.

The activity of water column material was studied by setting up "in situ" duplicate enrichments consisting of two hundred ml of water and 0.2 ml

Figure 1. Map showing location of sample sites used in this study (see Table 1 for description)

TABLE 1. SAMPLING LOCATIONS

Site No.	Description	Latitude (N)	Longitude (W)
1	Birch Bay State Park	485405	1224610
2	Between Atlantic Richfield and Mobil Docks (refinery site)	485108	1224500
3	Beach Access of Sucia Road	485124	1224349
4	Samish Island Public Beach	483450	1223235
5	2 miles east Jct. 20W and East March Point Road	482930	1223320
6	Mud Flats 2 miles from Jct. East March Point Road and East Fidalgo Roads	482930	1223550
7	Rosario Beach[a]	482510	1223935
8	Point Partridge Park	481330	1224605
9	Entrance Dungeness State Park	480935	1230900
10	Clallam Bay	481540	1241720
11	Pillar Point	481251	1240603
12	Salt Creek Recreation Campground	481000	1234217
13	Port Angeles (Below Red Lion Inn)	480710	1232525
14	Fort Worden State Park	480845	1224525
15	Jamestown	480831	1230623
16	Neah Bay	482337	1243021
17	Lopez Island - Shoal Bight[b]	482737	1224906
18	San Juan Island - South Beach[b]	482723	1230013
19	Orcas Island - Crescent Beach[b]	484141	1225351
20	Deception Pass State Park - West Beach	482408	1223946
21	Orcas Island - Terrill Beach[b]	484244	1225252
22	San Juan Island - False Bay[b]	482915	1230352

[a]only sampled in April and July, 1977; site 20 used for September, November, 1977 and January, 1978 surveys
[b]only sampled in September, 1977 survey

of Prudhoe Bay oil in 500 ml screw-capped Erlenmeyer flasks. One of the duplicate flasks received 2.0 ml of a nitrogen-phosphorus supplement. To permit aeration the screw-caps were replaced in the laboratory with sterile foam plugs prior to incubation on a rotary shaker. Ten grams of beach or sediment material were removed aseptically in a laminar flow hood and added to 500 ml Erlenmeyer flasks each containing 200 ml of sterile artificial sea water medium and 0.2 ml Prudhoe Bay oil. Duplicate flasks were prepared for each sample, one of which received a nutrient supplement of nitrogen and phosphorus, and all flasks were incubated in a rotary shaker. Unless otherwise specified all rotary shakers were run at 250 rpm and had an eccentricity of 3.8 cm. The temperature of incubation varied with experiments. After a suitable incubation period the oil was recovered and the pentane-soluble fraction subjected to gas chromatographic (G.C.) analysis. Activity was determined by comparing the G.C. profile of the saturate fraction of recovered oils with that of undegraded Prudhoe Bay oil subjected to the same recovery procedures. Typical G.C. profiles obtained by this technique and used in evaluating bacterial oil-degrading activity are shown in Figure 2. A "degradative capacity index" was calculated for each sample material by numerically rating a completely degraded n-saturate fraction (i.e. C) equal to 4; presence of residual isoprenoids (i.e. I) equal to 3; partial reduction of n-alkanes profile (i.e. P) equal to 2; selective removal of n-alkanes, C_{12} to C_{19} in carbon content (i.e. S) equal to 1; no degradation of n-alkanes (i.e. N) equal to 0; summing the numerical values and dividing by the number of samples.

Microbial Numbers

The spread plate technique using a basal marine agar was used in all these studies for determining total viable count. All dilution blanks used contained an artificial sea water salt solution at pH 7.3. Plates were dried before using by incubating for 2-3 days at 22°C and were stored at 4°C in sealed plastic bags. Plates used in the field were transported, inverted, in sealed plastic bags. Five replicate plates were prepared of each dilution and viable counts are reported as the average (±) one standard deviation. Microbial counts from water column samples are reported per ml and beach and sediment data per gram of dry material.

Isolates for taxonomic studies were obtained by grouping colonies growing in five plates of the dilution used for enumeration as to colonial color and morphology. Colonies representative of each type in all surveys were checked for purity by streaking on basal medium agar. The April isolates were examined for cellular morphology, Gram reaction, oxidase test and were compared as to their ability to grow in glucose tubes under aerobic and anaerobic conditions. The presence or absence of flagella on these isolates was determined by examining negatively-stained (1% phosphotungstic acid) preparations using an electron microscope. The isolates from the later surveys were examined for cellular morphology, Gram reaction, oxidase reaction, catalase reaction, and the utilization of glucose and lactose under aerobic and anaerobic conditions. Motility was determined either by examining wet mounts or by directly observing flagella with the electron microscope technique previously described.

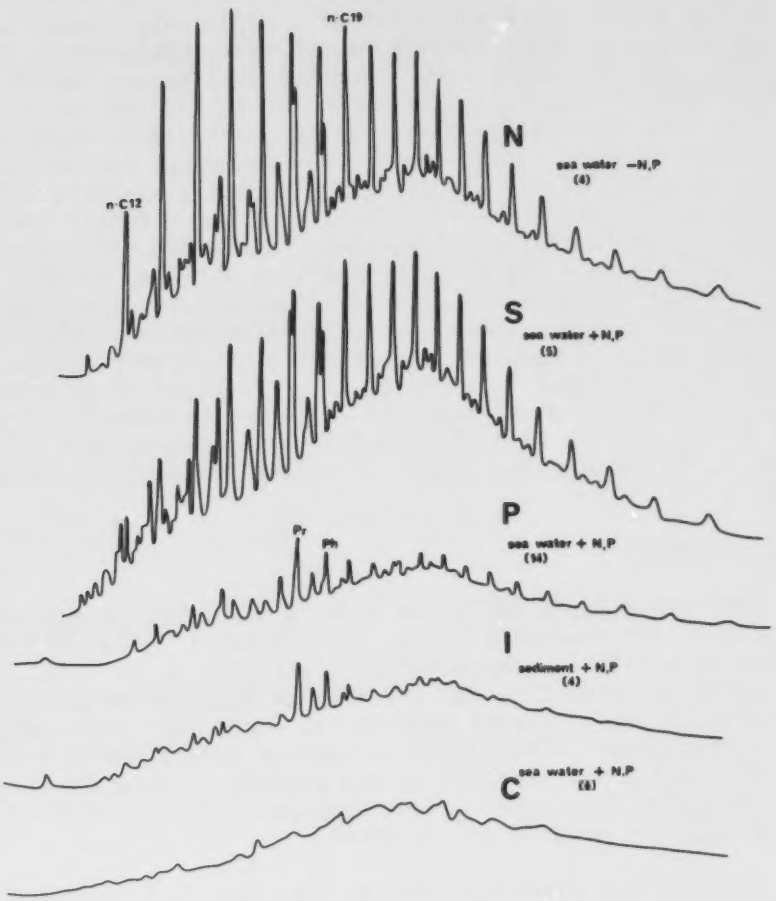

Figure 2. Typical G.C. profile of saturate fraction of Prudhoe Bay oil before and after growth of enrichment populations (N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P=partial removal, n-alkane peaks still discernible; I=only isoprenoids remaining; C=complete utilization of n-alkanes and isoprenoids).

Type cultures of isolates were kept by storage at 4°C on basal marine agar in sealed plastic bags. Some oil-degrading populations were maintained by monthly transfer in the enrichment medium containing Prudhoe Bay oil.

Physical and Chemical Measurements

All instruments were calibrated before and after each sample trip in addition to the usual adjustments made prior to each measurement.

The acidity of water column samples was determined using a portable Radiometer pH meter (Model #29) which was standardized using a Radiometer (51001) buffer system. Salinity and water column temperature were measured in the field using a Yellow Springs SCT meter (Model #33). The salinity function of this instrument was calibrated according to the manufacturer's specifications (i.e. with a standard solution of KCl in distilled water). Beach temperatures were obtained using a standard laboratory thermometer (0-50°C scale). Dissolved oxygen content of water column samples was obtained using a Yellow Springs Oxygen meter (Model #52) which was calibrated against a dropping mercury electrode system at the City of Edmonton's Goldbar Wastewater Treatment Plant analytical control laboratory.

Visual observations of habitat parameters were recorded and submitted as part of the computer data bank but are not included in this report.

The dry weights of beach and sediment samples were determined by accurately weighing, in triplicate, 1 to 2 gram samples and drying for 72 hours at 100°C. The average difference between the wet and dry weights was used to calculate a correction factor for reporting results on a dry weight basis. The weight of suspended material in water column samples was obtained by filtering aliquots through glass fibre filters (Reeve Angel) and weighing after drying to constant weight at 104°C.

The weight of oil lost due to weathering (inoculum consisted of sediment or water sterilized by autoclaving) and/or mineralization was determined by recovering oil from cultures using a chloroform extraction procedure and evaporating to dryness. This was followed by extraction of the residue with benzene, filtration of the soluble fraction, evaporation of the benzene and weighing of the dried residue. The sterility of the cultures used for measuring weathering was checked throughout the experiment using a plate count technique.

Oil was recovered from cultures for gas chromatography by a flotation technique. Cultures were cooled in an ice-water bath, and acidified to pH $\leq$ 1 to minimize emulsion formation. Five ml of n-pentane were added and the culture flasks were stoppered with rubber bungs previously washed with pentane and benzene. The inner surfaces of the flasks were gently rinsed with the pentane layer and then the contents mixed for 25 minutes on a rotary shaker at 200 rpm. The oil-pentane mixture was recovered by replacing the solid stopper with one containing a short fine bore glass tube (3 mm internal diameter) and a larger bore (5 mm internal diameter) glass tube reaching the bottom of the flask. Tap water was slowly added via the larger tube so that the pentane-oil mixture slowly floated to the top of the

Erlenmeyer flask and was recovered via the fine bore tube using a microlitre-hypodermic syringe. If a sample was not immediately run on the gas chromatograph, it was left in the acidified state and held at 4°C until the oil could be extracted and immediately analyzed.

The n-alkane and isoprenoid content of the pentane-soluble fraction of recovered oils was obtained on a Varian Aerograph Model 1740 gas chromatograph. This instrument is equipped with a flame ionization detector and two 6.1 m x 0.32 cm stainless steel columns packed with 3% SE 30 ultra-phase on Chromosorb W (AE-DMCS), 80/100 mesh. The carrier gas used was N_2 at a flow rate of 15 ml/min. The temperature program consisted of a 2 min holding period at 50°C, then the temperature was increased at a rate of 10°/min from 50°C to 300°C and program completed by a period of 20 min at 300°C.

The oxygen content of sealed incubation mixtures (used for radioactive studies) was determined using a Varian Model 700 gas chromatograph equipped with a thermal conductivity detector. Oxygen was resolved from other gases using a 3.7 m x 0.95 cm aluminum column packed with molecular sieve 13X (40/60 mesh). Helium was used as a carrier gas at a flow rate of 32 ml/min and the oven temperature was maintained at 60°C. Oxygen content was calculated using an air reference sample and comparing peak heights.

The methods used for nitrogen and phosphorus analyses are described in Methods of Soil Analysis (19). The ammonium and nitrate nitrogen content of samples was analyzed by a micro-Kjeldahl technique. Beach and sediment samples were dried and screened through a 2 mm sieve. The material passing through the sieve was divided into two samples: one was extracted with sodium bicarbonate (0.5M) and the other with an ammonium fluoride (0.03N)-dilute sulfuric acid (0.03N) solution. The soluble phosphorus content of these extracts and water column samples was determined by a colorimetric procedure utilizing an ammonium molybdate-ascorbic acid reagent.

Radioactivity Determinations

The liberation of $^{14}CO_2$ from the Prudhoe Bay oil "spiked" with either n-[1-^{14}C]-hexadecane or n-[^{14}C]-octadecane was used in studies aimed at determining the rate of oil degradation by marine microorganisms. Activity was measured using 125 ml Erlenmeyer flasks containing 20 µl of "spiked" oil and 25 ml of aqueous solution representing water column or 1:5 dilutions of beach or sediment material. The Erlenmeyer flasks were modified so that a small plastic cup containing a CO_2 trapping agent (either phenethylamine or Hyamine hydroxide) could be lowered into the air phase via the neck of the flask. A side port was added to the flask so that an acidifying agent (0.5 ml 4N H_2SO_4) could be injected into the flasks to stop growth and release CO_2. After a further 60 min incubation period the $^{14}CO_2$ content of the trapped CO_2 was determined in a toluene-based PPO/POPOP scintillation fluid using a Nuclear Chicago scintillation counter. When phenethylamine was used as the CO_2 trapping agent, radioactivity was measured in an Isocap Model 300 (unrefrigerated) scintillation counter. Chemiluminescence problems were encountered when Hyamine hydroxide was used for trapping CO_2 so when this absorbent was used, a Mark I scintillation counter (refrigerated) was used for determining the amount of radioactivity. Some of the problems

encountered in the application of this technique to rate studies are discussed in Appendix B.

Data Management

All the data collected or obtained by the physical-chemical and microbiological methods used in this study together with the characteristics of sample sites and types are available from the N.O.D.C., Washington, D.C.

SECTION 5

RESULTS

Survey for Activity of Oil-utilizing Bacteria

The physical-chemical parameters temperature, dissolved oxygen content and salinity of water column samples are presented in Tables D1, D2, D3 and D4 respectively. The pH of the majority of samples ranged from 6.6 to 8.3 and thus is suitable for normal heterotrophic bacterial growth. The temperatures which range from a low of 4.5°C in January, 1978 to a high of 16.0°C in September, 1977 would support psychrotrophic bacterial populations. Sufficient dissolved oxygen was present at all sampling times to support the presence of aerobic bacterial species. The salinity of April and September samples was in the range of 28 to 30 ppt with the lowest value being recorded in January which could have resulted from heavy December rains in this area.

The degradation states as shown by the G.C. profiles of the saturate fraction of Prudhoe Bay oil recovered after 28 days incubation at 8°C with water column, sediment and beach samples supplemented with added nitrogen and phosphorus are presented in Tables 2, 3 and 4 respectively. The pattern of n-alkane utilization observed varied both with sample site and sampling time. The results of similar experiments without nutrient supplementation are presented in Tables D5, D6 and D7. No degradation of the n-alkanes of Prudhoe Bay oil was observed without nutrient supplementation. The degradative patterns as a function of sample type and time of sampling are summarized in Table 5A, in terms of a "Degradative Capacity Index". The greatest variation in the degree of n-alkane utilization was observed with water column samples, the lowest value being found in January and highest in September samples. The lowest oil degrading activity for beach and sediment samples was observed in the September sampling. The beach samples showed the smallest variation in n-alkane utilization patterns observed over the three sampling periods. The slight activity found in non nutrient-enriched beach samples in September (Table D7) could be due to natural nutrient enrichment and/or the higher temperatures which occurred in this material during the summer months. The data in Table 5B show the summary of oil-degrading ability as a function of sample site. The greatest oil-degrading capability was observed in those sites closest to oil refinery activity i.e. the refinery site near Cherry Point and the E. Fidalgo Rd. site near Anacortes refineries, with slightly lower values being found at Pt. Angeles and Neah Bay. These outer sites are characterized by having a relatively high organic matter content and being subjected to chronic low levels of oil pollution (personal communication, Dr. D. Brown, NOAA Analytical Laboratories).

TABLE 2. G.C. PROFILE OF PENTANE EXTRACT OF RECOVERED PRUDHOE BAY OIL AFTER 28 DAYS INCUBATION AT 8°C WITH WATER COLUMN SAMPLES SUPPLEMENTED WITH NITROGEN AND PHOSPHORUS

Site	G.C. Profile[a]		
	April '77	Sept. '77	Jan. '78
Birch Bay[b]	P	P	N
Refinery Site	S	C	I
Sandy Pt. (Sucia Rd.)	I	I	P
Samish Island (Public Beach)	I	I	N
March Pt. Rd.	S	I	S
E. Fidalgo Rd.	C	I	S
Deception Pass[b]	C	I	N
Pt. Partridge[b]	S	I	S
Ft. Worden[b]	P	C	N
Jamestown	-[d]	C	P
Dungeness	P	P	S
Pt. Angeles	P	P	I
Salt Creek[b]	P	-	-
Pillar Pt.	I	P	P
Clallam Bay	S	P	S
Neah Bay	-	P	S
Lopez Island[c]			
Shoal Bight	-	C	-
San Juan Island[c]			
South Beach	-	C	-
False Bay	-	I	-
Orcas Island[c]			
Crescent Beach	-	C	-
Terrill Beach	-	C	-

[a]N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P=partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids.
[b]State Park
[c]only sampled in September, 1977
[d]no sample

TABLE 3. G.C. PROFILE OF PENTANE EXTRACT OF RECOVERED PRUDHOE BAY OIL AFTER 28 DAYS INCUBATION AT 8°C WITH SEDIMENT SAMPLES SUPPLEMENTED WITH NITROGEN AND PHOSPHORUS

	G.C. Profile[a]		
Site	April '77	Sept. '77	Jan. '78
Birch Bay[b]	I	N	S
Refinery Site	I	P	-
Sandy Pt. (Sucia Rd.)	I	S	P
Samish Island (Public Beach)	I	S	S
March Pt. Rd.	I	-	I
E. Fidalgo Rd.	I	P	C
Deception Pass[b]	I	I	N
Pt. Partridge[b]	S	N	N
Ft. Worden[b]	I	N	I
Jamestown	-[d]	P	S
Dungeness	I	N	-
Pt. Angeles	P	I	I
Salt Creek[b]	-	-	-
Pillar Pt.	I	I	-
Clallam Bay	I	P	S
Neah Bay	-	I	I
Lopez Island[c]			
Shoal Bight	-	P	-
San Juan Island[c]			
South Beach	-	N	-
False Bay	-	P	-
Orcas Island[c]			
Crescent Beach	-	N	-
Terrill Beach	-	P	-

[a]N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P=partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids.
[b]State Park
[c]only sampled in September, 1977
[d]no sample

TABLE 4. G.C. PROFILE OF PENTANE EXTRACT OF RECOVERED PRUDHOE BAY OIL AFTER 28 DAYS INCUBATION AT 8°C WITH BEACH SAMPLES SUPPLEMENTED WITH NITROGEN AND PHOSPHORUS

	G.C. Profile[a]		
Site	April '77	Sept. '77	Jan. '78
Birch Bay[b]	I	S	S
Refinery Site	C	I	C
Sandy Pt. (Sucia Rd.)	I	P	I
Samish Island (Public Beach)	S	P	S
March Pt. Rd.	I	I	-
E. Fidalgo Rd.	C	I	C
Deception Pass[b]	I	P	I
Pt. Partridge	I	P	S
Ft. Worden[b]	P	N	I
Jamestown	-[d]	C	P
Dungeness	I	P	-
Pt. Angeles	I	I	I
Salt Creek[b]	-	-	-
Pillar Pt.	I	P	-
Clallam Bay	I	N	P
Neah Bay	-	C	I
Lopez Island[c]			
Shoal Bight	-	S	-
San Juan Island[c]			
South Beach	-	I	-
False Bay	-	I	-
Orcas Island[c]			
Crescent Beach	-	N	-
Terrill Beach	-	P	-

[a]N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P=partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids.
[b]State Park
[c]only sampled in September, 1977
[d]no sample

TABLE 5. SUMMARY OF OIL-DEGRADING BACTERIAL ACTIVITY AT 8°C

(A) In Water Column, Sediment and Beach Samples Respectively

Sample	Nitrogen Phosphorus Supplementation	Degradative Capacity Index[a] April '77	Sept. '77	Jan. '78
Water	+	2.28	3.05	1.2
	-	0	0	0
Sediment	+	2.78[b]	1.47	1.8
	-	0	0	0
Beach	+	2.92[b]	2.10	2.5
	-	0	0	0

(B) At Sample Sites

Site	Degradative Capacity Index[a]	Site	Degradative Capacity Index[a]
Birch Bay[c]	1.44	Salt Creek	—[e]
Refinery Site	3.00	Pillar Pt.	2.57
Sandy Pt. (Sucia Rd.)	2.32	Clallam Bay	1.67
Samish Island (Public Beach)	1.67	Neah Bay	2.67
March Pt. Rd.	2.42	Lopez Island[d]	
E. Fidalgo Rd.	3.11	Shoal Bight	2.00
Deception Pass[c]	2.33	San Juan Island[d]	
Pt. Partridge[c]	1.34	South Beach	2.34
Ft. Worden[c]	1.89	False Bay	2.60
Jamestown	2.50	Orcas Island[d]	
Dungeness	1.86	Crescent Beach	1.00
Pt. Angeles	2.67	Terrill Beach	2.34

[a] a value of 4.0 would be obtained if all samples in a survey completely utilized the n-alkanes and isoprenoids in Prudhoe Bay oil. (See Materials and Methods)
[b] values of 3.15 and 3.46 for sediment and beach samples supplemented with nitrogen and phosphate incubated for 14 days at 30°C
[c] State Park
[d] only sampled in September, 1977
[e] only one water column sample obtained at this site (April, 1977)

Bacterial Numbers and Oil-degrading Activity

A comparison of the bacterial counts of the April sediment and beach samples with the degradative pattern observed (Table 6) indicates that there is no positive relationship between viable bacterial counts and degree of utilization of n-alkane components of Prudhoe Bay oil. Viable bacterial populations of water column samples ranged from 10^3 to 10^4 cells per ml for "pristine" areas like Pt. Partridge Park to 10^5 to 10^6 for waters adjacent to oil refinery activities such as the refinery and E. Fidalgo Rd. sites. A ten-fold increase in viable population was observed in water column samples shipped by air from sites examined.

Factors Affecting Oil Degradation

(a) Temperature

The effect of incubation temperature on the utilization of Prudhoe Bay oil by microorganisms present in the beach and sediment samples obtained in the April survey is shown by comparing data in Table 7 (i.e. at 30°C) with those in Tables 3 and 4 (i.e. at 8°C). A much greater activity is observed at the higher temperature as the "Degradative Capacity Index" for the sediment and beach samples incubated at 30°C is 3.15 and 3.75 respectively compared with 2.78 and 2.92 for the 8°C incubations. The 30°C data in this experiment also was obtained with only one-half (i.e. 14 days) the incubation time of that used at 8°C.

The effect of incubation temperatures on changes brought about in the n-alkane components of Prudhoe Bay oil with sediment from March Pt. Rd. (i.e. Anacortes refinery area) and the Deception Pass sites (Rosario Beach - a "pristine" environment) as a function of incubation time is shown in Table 8. The rate of utilization of n-alkanes, as observed earlier, increases as the incubation temperature is raised. The microbial populations in the sediment from the refinery area (March Pt. Rd.) bring about more rapid changes in the n-alkane profile than do the populations in sediment from Rosario Beach. A more detailed study (Table D8) shows the changes in total viable count at 8°C and 20°C and the accompanying changes in the G.C. profile of recovered oil, confirming the previous observations.

The effect of incubation temperature on the generic components of the indigenous bacterial populations in water column and sediment samples from West Beach (Deception Pass) is presented in Table 9. Differences between the percentage generic incidence within water column and sediment samples and between temperatures of incubation within the same sample material are readily discernible. However no relationship between generic composition and differences in rate of oil utilization is apparent.

(b) Nitrogen and Phosphorus Levels

The effect of the level of nitrogen and phosphorus supplementation on the degradative capacity of water column samples from March Pt. Rd. and West Beach (Deception Pass) is shown in Table 10. The results indicate that the degradative pattern obtained is more sensitive to nitrogen deprivation

TABLE 6. VIABLE BACTERIAL COUNTS OF BEACH AND SEDIMENT SAMPLES OBTAINED IN APRIL, 1977 (28 DAYS INCUBATION at 8°C)

Site	$\times 10^4/g$[a] Sediment		Beach	
Birch Bay[b]	16.6 ± 6.2	(I)[c]	59 ± 22.0	(I)
Refinery Site	9.9 ± 1.9	(I)	2.8 ± 0.4	(C)
Sandy Pt. (Sucia Rd.)	1.0 ± 0.04	(I)	106 ± 15.7	(I)
Samish Island (Public Beach)	126 ± 21.6	(I)	25.6 ± 4.9	(S)
March Pt. Rd.	52 ± 7.1	(I)	13.2 ± 2.1	(I)
E. Fidalgo Rd.	161 ± 17.4	(I)	17.8 ± 3.8	(C)
Deception Pass[b]	12.9 ± 2.0	(I)	35 ± 11.5	(I)
Pt. Partridge	7.0 ± 0.7	(S)	0.6 ± 0.01	(I)
Ft. Worden[b]	5.6 ± 1.6	(I)	25.0 ± 1.7	(P)
Dungeness	33.6 ± 5.5	(I)	65 ± 10.1	(I)
Pt. Angeles	62 ± 1.5	(P)	21.8 ± 3.0	(I)
Pillar Pt.	106 ± 21.2	(I)	168 ± 38.3	(I)
Clallam Bay	0.7 ± 0.0	(I)	61 ± 12.4	(I)

[a]average ± one standard deviation
[b]State Park
[c]G.C. profile (S=selective metabolism, n-alkanes C_{12} to C_{19}; P= partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids).

TABLE 7. G.C. PROFILE OF PENTANE EXTRACT OF RECOVERED PRUDHOE BAY OIL AFTER 14 DAYS INCUBATION AT 30°C WITH BEACH AND SEDIMENT SAMPLES[a]

	G.C. Profile[b]	
Site	Sediment	Beach
Birch Bay[c]	I	C
Refinery Site	I	C
Sandy Pt. (Sucia Rd.)	I	-
Samish Island (Public Beach)	P	C
March Pt. Rd.	C	C
E. Fidalgo Rd.	I	C
Deception Pass[c]	C	C
Pt. Partridge[c]	P	I
Ft. Worden[c]	P	I
Dungeness	C	C
Pt. Angeles	C	C
Pillar Pt.	C	C
Clallam Bay	I	I

[a]samples taken in April survey (nitrogen and phosphorus added)
[b]P=partial removal, n-alkane peaks still discernible; I= only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids
[c]State Park
[d]no sample

TABLE 8. EFFECT OF TEMPERATURE AND INCUBATION TIME ON VIABLE BACTERIAL COUNTS AND ON THE G.C. PROFILE OF RECOVERED PRUDHOE BAY OIL INCUBATED WITH SEDIMENT[a] FROM MARCH PT. RD. AND DECEPTION PASS (ROSARIO BEACH)

		March Pt. Rd.		Rosario Beach	
Temperature	Time (days)	G.C. profile[b]	viable bacterial count $x10^6/g$[c]	G.C. profile[b]	viable bacterial count $x10^6/g$[c]
8°C	4	N	10.6 ± 1.4	N	0.8 ± 0.0
	7	-[d]	-	N	1.8 ± 0.1
	15	S	16.3 ± 2.6	N	7.4 ± 0.4
	29	I	26.2 ± 1.7	P	66 ± 14.3
14°C	4	N	7.5 ± 0.8	N	3.1 ± 0.3
	7	N	19.2 ± 2.5	N	-
	15	P	39 ± 6.6	S	75 ± 15.6
	29	I	47.9 ± 5.1	C	127 ± 29.3
20°C	4	N	11.5 ± 1.7	N	4.5 ± 0.4
	7	I	56 ± 12.4	N	44 ± 6.1
	15	I	116 ± 9.2	P	285 ± 79.3
	29	C	34 ± 6.2	-	-
30°C	4	I	245 ± 34.2	I	233 ± 43.8
	7	I	121 ± 14.7	I	166 ± 12.5
	15	I	70 ± 15.9	I	154 ± 38.4
	29	C	39 ± 7.2	-	-

[a] sites sampled in July, 1977

[b] N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P= partial removal, n-alkanes still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids

[c] average ± one standard deviation

[d] no sample

TABLE 9. EFFECT OF INCUBATION TEMPERATURE ON GENERIC COMPOSITION OF BACTERIAL POPULATIONS IN WATER COLUMN AND SEDIMENT SAMPLES (SEPT., 1977) FROM WEST BEACH (DECEPTION PASS)

	% Generic Composition			
	Water Column		Sediment	
Genus	8°C	20°C	8°C	20°C
Alcaligenes	3.2 (1)[a]	-[b]	18.5 (1)	-
Bacillus	-	-	11.1 (1)	-
coryneforms	3.8 (1)	9.4 (3)	-	-
Cytophaga	4.1 (3)	0.8 (1)	9.3 (2)	10.8 (3)
Flavobacterium	44.4 (6)	26.7 (5)	3.8 (1)	30.0 (3)
Pseudomonas	24.4 (3)	25.6 (4)	27.8 (4)	39.2 (3)
Vibrio	4.1 (1)	-	-	-
Unidentified[c]	15.0 (1)	37.6 (2)	20.4 (2)	13.3 (2)
Non-transferable colonies[d]	0.9 (2)	-	9.3 (1)	6.7 (1)

[a]number of colonial types in parentheses
[b]no colonies appeared on dilution plate used to assess population composition
[c]similar to either Alcaligenes or Pseudomonas spp. with the exception that isolates were capable of non acid-generating, anaerobic growth
[d]colonies would not grow on transfer

TABLE 10. EFFECT OF NITROGEN AND PHOSPHORUS CONCENTRATION ON THE G.C. PROFILE[a] OF THE N-SATURATE FRACTION OF RECOVERED PRUDHOE BAY OIL AFTER 14 DAYS INCUBATION AT 20°C WITH WATER COLUMN SAMPLES FROM MARCH PT. RD. AND WEST BEACH (DECEPTION PASS)[b]

		Phosphorus				
March Pt. Rd.		2N	N[c]	0.1N	0.01N	0
Nitrogen	2N	C	C	C	C	P
	N[c]	C	-	I	C	P
	0.1N	C	S	C	C	P
	0.01N	P	N	P	P	P
	0	N	N	N	N	N
West Beach (Deception Pass)						
Nitrogen	2N	C	C	P	I	S
	N[c]	S	N	C	P	S
	0.1N	C	S	I	I	P
	0.01N	S	N	P	P	N
	0	N	N	S	N	N

[a]N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P=partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids

[b]samples obtained in November, 1977

[c]normal levels used in all enrichment and survey studies equivalent to 350 µg N per ml and 180 µg P per ml

than to low phosphorus supplementation. Similar results were obtained in a study using a combined sediment sample from E. Fidalgo Rd. and Neah Bay (Table 11). This effect is supported by the results of the chemical analysis of samples for nitrogen and phosphorus (Table D9). Phosphorus was detected in all samples tested whereas nitrogen levels were below the sensitivity of the method used in the survey. Increasing the nitrogen and phosphorus levels beyond those normally used in the survey resulted in a more rapid change in the physical appearance of such cultures when compared to those receiving a normal nutrient supplement.

(c) Oil Concentration

The data presented in Table 12 indicate that oil concentration has an effect on the rate of utilization of the n-alkanes present in Prudhoe Bay oil particularly at 8°C. The beach samples from the "pristine" Rosario site used in this experiment were not as effective in yielding psychrotrophic oil-degrading bacteria as was the sample from the refinery site, i.e. March Pt. Rd.

Rate of Oil Utilization

Gravimetric data on the rate of weight loss of Prudhoe Bay oil, under laboratory conditions, are presented in Table 13. Approximately 30 to 34% of the oil was lost due to "weathering" (i.e. physical, chemical processes), 35% due to mineralization (i.e. bacterial action) and approximately 30 to 35% of the original weight of oil was left as a residue. Further incubation of up to 48 days did not result in any significant changes in the loss of oil due to "weathering" or mineralization. Similar experiments were carried out with sediment samples from these two sites. However, while similar patterns were observed, considerable variation in replicates was noted.

As the gravimetric procedure is not very sensitive, a radioactive technique was used which measured the rate of $^{14}CO_2$ evolution from Prudhoe Bay oil "spiked" with carbon-14 labelled hexa- or octadecane. The results obtained with the water column samples from March Pt. Rd. and West Beach (Deception Pass) are presented in Figure 3. Considerable scattering was observed particularly with the sample from March Pt. Rd. This was attributed in part to the presence of a high content of suspended material (Table D10) of the March Pt. Rd. sample and the failure during sub-sampling of this material to keep the particles in suspension. A rate of 0.42 and 0.12 mg hexadecane was removed per litre per day during the linear phase of $^{14}CO_2$ evolution for March Pt. Rd. and West Beach (Deception Pass) water column samples supplemented with nitrogen and phosphorus. A similar experiment (Figure D1) with non nutrient-supplemented water samples yielded initial rates of $^{14}CO_2$ release corresponding to the utilization of 0.008 mg and 0.060 mg of hexadecane per litre per day. However the G.C. profile of the substrate fraction of recovered oils from the non-nutrient supplemented samples revealed no detectable changes in the n-alkane profile of recovered oil although 30,000 DPM were recovered from the West Beach (Deception Pass) sample. The re-utilization of produced $^{14}CO_2$ is indicated in both experiments but only with nutrient supplementation does enough growth take place to markedly change the n-alkane profile of recovered oil. The results from a similar experiment using sediment material from March Pt. Rd. and West Beach (Deception Pass)

TABLE 11. EFFECT OF NITROGEN AND PHOSPHORUS CONCENTRATIONS ON THE G.C. PROFILE[a] OF THE N-SATURATE FRACTIONS OF PRUDHOE BAY OIL AFTER 14 DAYS INCUBATION AT 20°C WITH SEDIMENT (SAMPLE WAS A MIXTURE OF SEDIMENT FROM E. FIDALGO RD. AND NEAH BAY)[b]

		Phosphorus						
		4N	2N	N[c]	0.1N	0.05N	0.01N	0
Nitrogen	4N	C	I	C	P	I	I	P
	2N	C	C	C	I	I	I	S
	N[c]	I	C	C	C	I	I	P
	0.1N	C	C	C	I	I	I	P
	0.05N	P	I	-	P	P	P	N
	0.01N	N	S	N	N	S	N	N
	0	S	S	-	S	N	N	N

[a]N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P=partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids

[b]samples obtained in September, 1977

[c]normal levels in all enrichment and survey studies equivalent to 350 µg N per ml and 180 µg P per ml

TABLE 12. EFFECT OF OIL CONCENTRATION ON GROWTH AND G.C.[a] PATTERN OF SATURATE FRACTIONS OF RECOVERED PRUDHOE BAY OIL.

Temperature (days incubation)	Oil[b]	Beach Samples[c]			
		March Pt. Rd.		Rosario	
		G.C. profile	x10^6[d]	G.C. profile	x10^6
8°C (29)	500	C	40 ± 4.7	N	16.5 ± 1.6
	1000	I	56 ± 8.1	-	—[e]
	2000	S	67 ± 9.7	N	70 ± 11.0
	4000	N	87 ± 19.0	N	187 ± 13.7
	8000	N	111 ± 29.5	N	278 ± 18.9
20°C (14)	500	C	40.1 ± 8.9	C	550 ± 129
	1000	C	124 ± 21.5	-	—
	4000	C	730 ± 8.3	C	1360 ± 115
	8000	C	3350 ± 509	P	1390 ± 414

[a]N=no degradation; S=selective metabolism, n-alkane C_{12} to C_{19}; P= partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids
[b]concentration to nearest 10 mg/500 ml culture medium
[c]samples obtained in July, 1977
[d]average viable count/ml ± one standard deviation
[e]sample lost

TABLE 13. WEATHERING AND MINERALIZATION OF PRUDHOE BAY OIL-WATER COLUMN SAMPLES[a] FROM MARCH PT. RD. AND ROSARIO BEACH SUPPLEMENTED WITH NITROGEN AND PHOSPHORUS INCUBATED AT 8°C AND 20°C

Time (days)	8°C				20°C			
	March Pt. Rd.		Rosario Beach		March Pt. Rd.		Rosario Beach	
	% weathered[b]	% mineralized[c]	% weathered	% mineralized	% weathered	% mineralized	% weathered	% mineralized
5	-[d]	-	-	-	30.6	2.1 (I)	30.5	14.3 (I)
7	31.8	0.1 (N)[e]	30.2	0.9 (N)	-	-	-	-
8	-	-	-	-	33.6	26.0 (I)	30.1	39.5 (C)
13	-	-	-	-	34.0	20.7 (C)	32.5	34.9 (C)
14	34.0	6.3 (I)	33.4	3.7 (S)	-	-	-	-
20	-	-	-	-	32.4	33.0 (C)	33.6	32.5 (C)
21	33.0	23.4 (C)	33.6	21.2 (C)	-	-	-	-
27	-	-	-	-	-	-	-	-
28	30.4	39.0 (C)	30.8	35.5 (C)	-	-	-	-

[a]samples obtained in July, 1977
[b]weight loss of uninoculated controls; each flask contained 0.1982 to 0.2141 gms of oil/200 ml medium.
[c]weight loss as a result of microbial activity
[d]no sample
[e]G.C. profile (N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P= partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids)

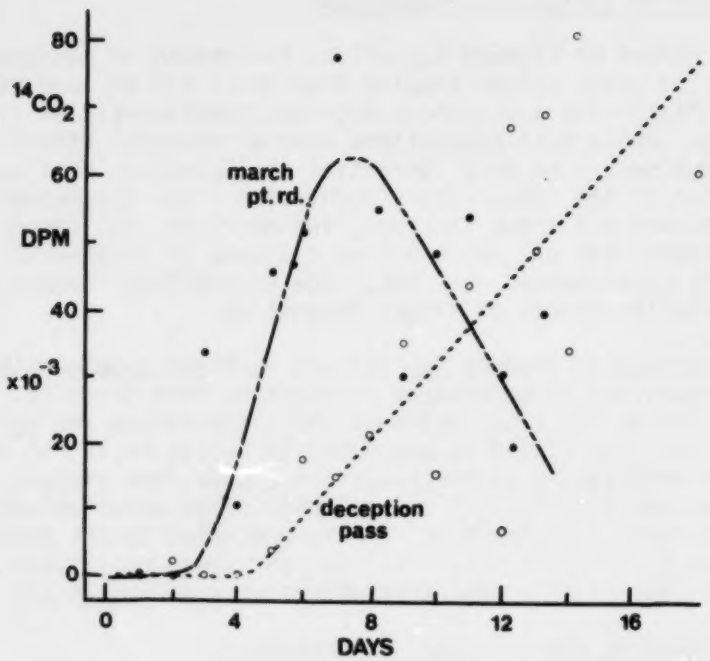

Figure 3. Rate of $^{14}CO_2$ release from Prudhoe Bay oil containing ^{14}C-hexadecane incubated with water column samples from March Pt. Rd. and West Beach (Deception Pass) supplemented with nitrogen and phosphorus (DPM=disintegrations per minute).

are presented in Figure 4. A rate of 3.4 mg hexadecane removed per kg of sediment per day during the linear phase of $^{14}CO_2$ evolution for March Pt. Rd. and West Beach (Deception Pass) sediment samples was calculated.

The results presented in Figure 5, using a slightly different radioactivity technique, show a rate of $^{14}CO_2$ liberation from ^{14}C-octadecane containing Prudhoe Bay oil of 1.1 mg octadecane per kg sediment per day (mixed sample from Jamestown and Ft. Worden sites after two months' storage at -20°C). The plot represents the average of five replicates plus or minus one standard deviation and shows an acceptable degree of reproducibility.

Effect of Oil on Indigenous Population

The effect of Prudhoe Bay oil on the growth of indigenous bacterial populations of water column samples from March Pt. Rd. and West Beach (Deception Pass) with and without nutrient supplementation at 20°C is shown in Figure 6. While both populations show a "diauxie" effect (two successive growth cycles separated by a lag phase) the Deception Pass sample also shows an inhibition of the total viable population. Gas chromatographic analysis of the recovered oil shows that only the March Pt. Rd. sample yielded a residual Prudhoe Bay oil which had no n-alkane or isoprenoid components. The nutrient-supplemented West Beach (Deception Pass) sample also would have yielded a similar result on longer incubation.

The effect of Prudhoe Bay oil and nutrient supplementation on the generic composition of enrichment populations from March Pt. Rd. beach, West Beach (Deception Pass) sediment and water column and sediment from False Bay, San Juan Island is presented in Tables 14, 15, 16 and 17 respectively. The generic composition of the bacterial populations present in beach and sediment samples (April, 1977 survey) and in the water column samples (September, 1977) from March Pt. Rd. is presented in the Appendix in Tables D11, D12 and D13 respectively. All oil-enriched populations contain a significant content of either coryneforms or Pseudomonas spp.

Storage of Samples and Oil-degrading Capability

The data presented in Figure 7 show the changes in viable cell count for sediment samples from March Pt. Rd. and West Beach (Deception Pass) stored at different temperatures. All samples after 6 weeks storage were still capable of bringing about the complete utilization of the n-alkane fraction of Prudhoe Bay oil. Changes were noted in the colonial characteristics of the viable population as the proportion of pigmented colonies decreased after storage at 4°C. Similar viability patterns were observed, although of a different magnitude, with both sediment sample types. Growth took place in both samples stored at 4°C with slight decreases being observed between the 1st and 2nd week of storage at -20°C and -60°C.

Although water column samples increased in viable cell number during transit such samples after a further 12 days storage at 4°C still retained the capability of utilizing the n-alkanes and isoprenoids present in Prudhoe Bay oil.

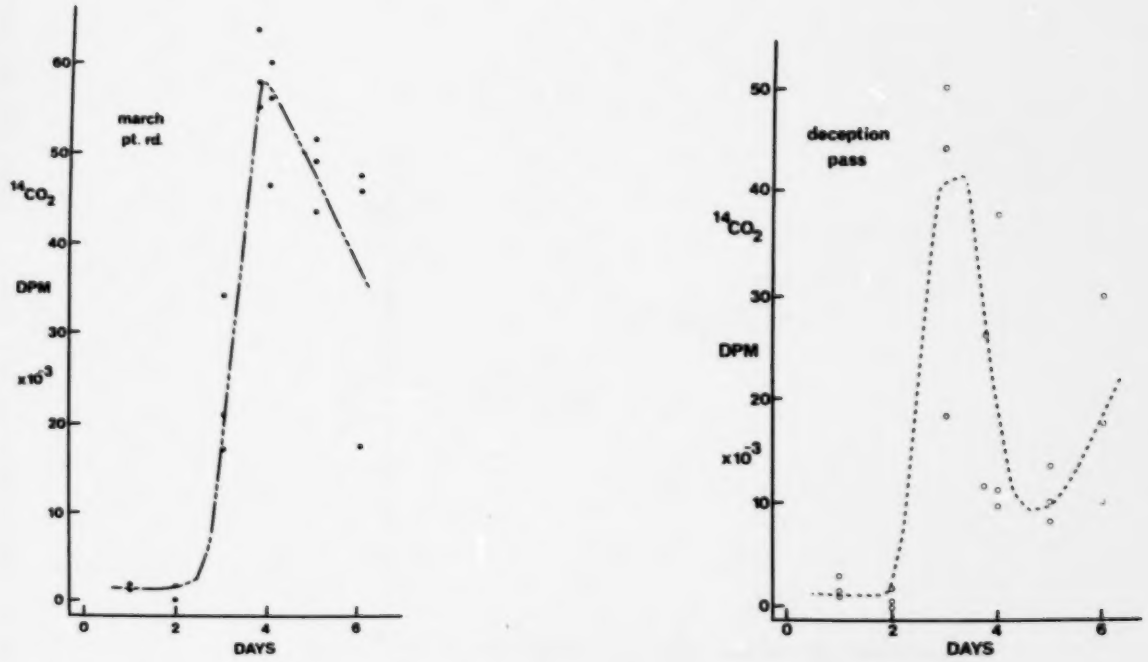

Figure 4. Rate of $^{14}CO_2$ release from Prudhoe Bay oil containing ^{14}C-hexadecane incubated with sediment samples from March Pt. Rd. and West Beach (Deception Pass) supplemented with nitrogen and phosphorus.

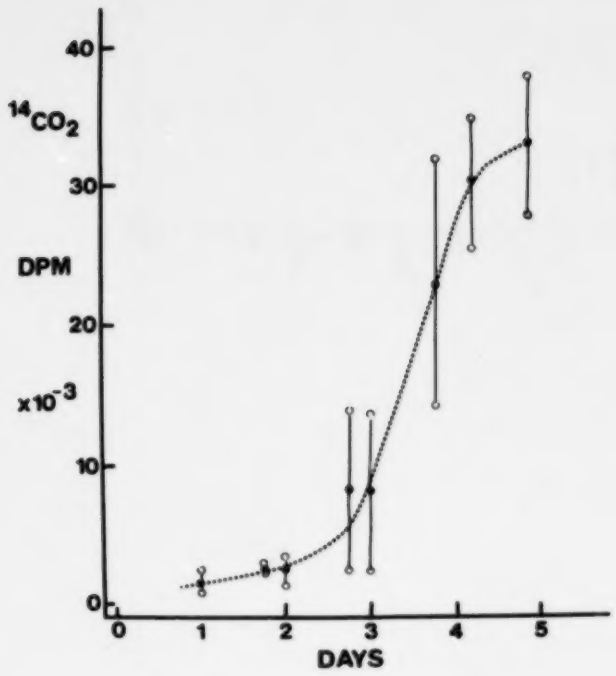

Figure 5. Graph of time of incubation vs net dpm ± 1 standard deviation from sediment (mixed sample from Jamestown and Ft. Worden taken in January, 1978).

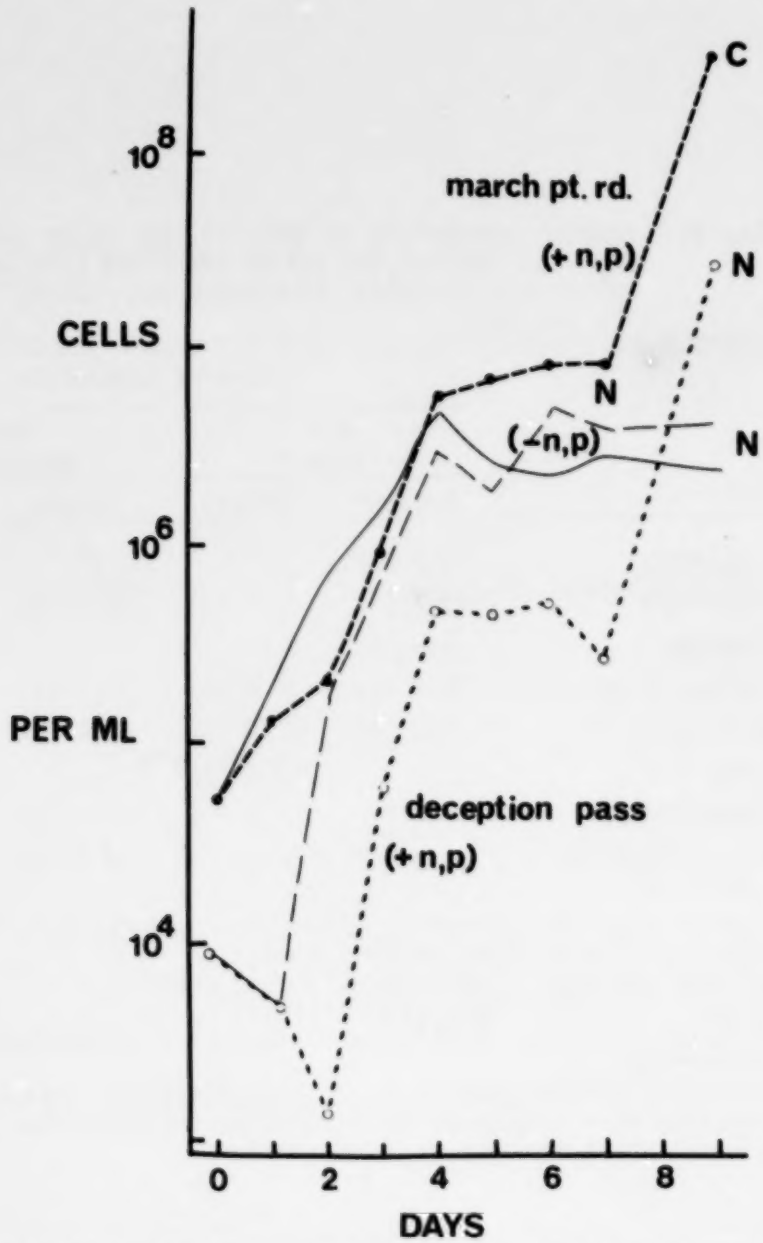

Figure 6. Effect of Prudhoe Bay oil on the growth (20°C) of bacteria present in March Pt. Rd. and West Beach (Deception Pass) water column samples (Nov., 1977) with and without nitrogen, phosphorus supplementation. (March Pt. Rd. ●---● +N.P., —— -N.P.; West Beach (Deception Pass) o----o +N.P., — — — -N.P.). Description of G.C. profile, n-alkane components, saturate fraction of recovered oil; N=no change, C=complete utilization of n-alkanes and isoprenoids.

TABLE 14. GENERIC COMPOSITION OF MARCH PT. RD. BEACH (SEPT., 1977) BACTERIAL POPULATIONS BEFORE AND AFTER ENRICHMENT IN THE PRESENCE OF NITROGEN, PHOSPHORUS AND PRUDHOE BAY OIL AT 8°C and 20°C

Genus	% Generic Composition			
	8°C enrichment		20°C enrichment	
	Before[a]	After[b]	Before	After
Alcaligenes	5.7 (1)[c]	-	-[d]	-
coryneforms	29.7 (4)	22.8 (2)	40.4 (6)	52.4 (5)
Cytophaga	2.3 (3)	-	-	0.3 (1)
Flavobacterium	58.8 (8)	34.8 (5)	30.3 (3)	42.1 (3)
Pseudomonas	2.3 (3)	12.0 (1)	24.9 (6)	-
Vibrio	-	8.9 (1)	-	-
Unidentified[e]	1.1 (1)	21.5 (6)	-	5.2 (2)
Non-transferable colonies[f]	-	-	4.3 (1)	-

[a]before enrichment (i.e. original sample)
[b]nitrogen, phosphorus and Prudhoe Bay oil present
[c]number of colonial types in parentheses
[d]no colonies present on dilution plate used to assess population composition
[e]similar to either Alcaligenes or Pseudomonas spp. with the exception that they were capable of non acid-generating anaerobic growth
[f]colonies which would not grow on transfer

TABLE 15. GENERIC COMPOSITION OF BACTERIAL POPULATIONS IN SEDIMENT SAMPLES (SEPT., 1977) FROM WEST BEACH (DECEPTION PASS) BEFORE AND AFTER ENRICHMENT IN THE PRESENCE OF NITROGEN, PHOSPHORUS AND PRUDHOE BAY OIL

Genus	% Generic Composition			
	8°C enrichment		20°C enrichment	
	Before[a]	After[b]	Before	After
Alcaligenes	18.5 (1)[c]	5.2 (1)	-[d]	-
Bacillus	11.1 (1)	-	-	-
coryneforms	-	12.7 (2)	-	31.7 (4)
Cytophaga	9.3 (2)	-	10.8 (3)	-
Flavobacterium	3.8 (1)	8.0 (1)	30.0 (3)	35.5 (1)
Pseudomonas	27.8 (4)	50.9 (2)	39.2 (3)	32.8 (4)
Vibrio	-	7.1 (2)	-	-
Unidentified[e]	20.4 (2)	16.0 (4)	13.3 (2)	-
Non-transferable colonies[f]	9.3 (1)	-	6.7 (1)	-

[a]before enrichment (i.e. original sample)
[b]nitrogen, phosphorus and Prudhoe Bay oil
[c]number of colonial types in parentheses
[d]no colonies present in dilution plate used to assess population composition
[e]similar to either Alcaligenes or Pseudomonas spp. with the exception that isolates were capable of non acid-generating anaerobic growth
[f]colonies would not grow on transfer

TABLE 16. GENERIC COMPOSITION OF BACTERIAL POPULATIONS IN WATER COLUMN SAMPLES (SEPT., 1977) FROM WEST BEACH (DECEPTION PASS) BEFORE AND AFTER ENRICHMENT ON THE PRESENCE OF NITROGEN, PHOSPHORUS AND PRUDHOE BAY OIL

Genus	% Generic Composition			
	8°C enrichment		20°C enrichment	
	Before[a]	After[b]	Before	After
Alcaligenes	3.2 (1)[c]	-[d]	-	0.4 (1)
coryneforms	3.8 (1)	8.7 (3)	9.4 (3)	34.8 (3)
Cytophaga	4.1 (3)	-	0.8 (1)	-
Flavobacterium	44.4 (6)	5.8 (3)	26.7 (5)	19.5 (3)
Pseudomonas	24.4 (3)	48.0 (2)	25.6 (4)	45.3 (5)
Vibrio	4.1 (1)	0.7 (1)	-	-
Unidentified[e]	15.0 (1)	39.8 (5)	37.6 (2)	-
Non-transferable colonies[f]	0.9 (2)	-	-	-

[a]before enrichment (i.e. original sample)
[b]nitrogen, phosphorus and Prudhoe Bay oil present
[c]number of colonial types in parentheses
[d]no colonies present in dilution plate used to assess population composition
[e]similar to either Alcaligenes or Pseudomonas spp. with the exception that isolates were capable of non acid-generating, anaerobic growth
[f]colonies would not grow on transfer

TABLE 17. GENERIC COMPOSITION OF BACTERIAL POPULATIONS IN SEDIMENT (SEPT., 1977) FROM FALSE BAY, SAN JUAN ISLAND BEFORE AND AFTER ENRICHMENT IN THE PRESENCE OF NITROGEN, PHOSPHORUS AND PRUDHOE BAY OIL AT 8°C AND 20°C

Genus	% Generic Composition			
	8°C enrichment		20°C enrichment	
	Before[a]	After[b]	Before	After
Alcaligenes	19.0 (1)[c]	34.8 (2)	18.0 (1)	56.1 (3)
coryneforms	0.3 (1)	16.6 (3)	-[d]	5.4 (2)
Cytophaga	1.1 (1)	-	0.1 (1)	-
Flavobacterium	47.1 (2)	19.1 (2)	63.6 (3)	1.8 (2)
Pseudomonas	2.5 (2)	-	4.0 (3)	37.8 (6)
Vibrio	2.2 (1)	3.5 (1)	-	-
Unidentified[e]	24.4 (5)	26.1 (1)	4.9 (2)	-
Non-transferable colonies[f]	3.4 (1)	-	9.4 (1)	-

[a]before enrichment (i.e. original sample)
[b]nitrogen, phosphorus and Prudhoe Bay oil present
[c]number of colonial types in parentheses
[d]no colonies present on dilution plate used to assess population composition
[e]similar to either Alcaligenes or Pseudomonas spp. with the exception that colonies were capable of non acid-generating, anaerobic growth
[f]colonies would not grow on transfer

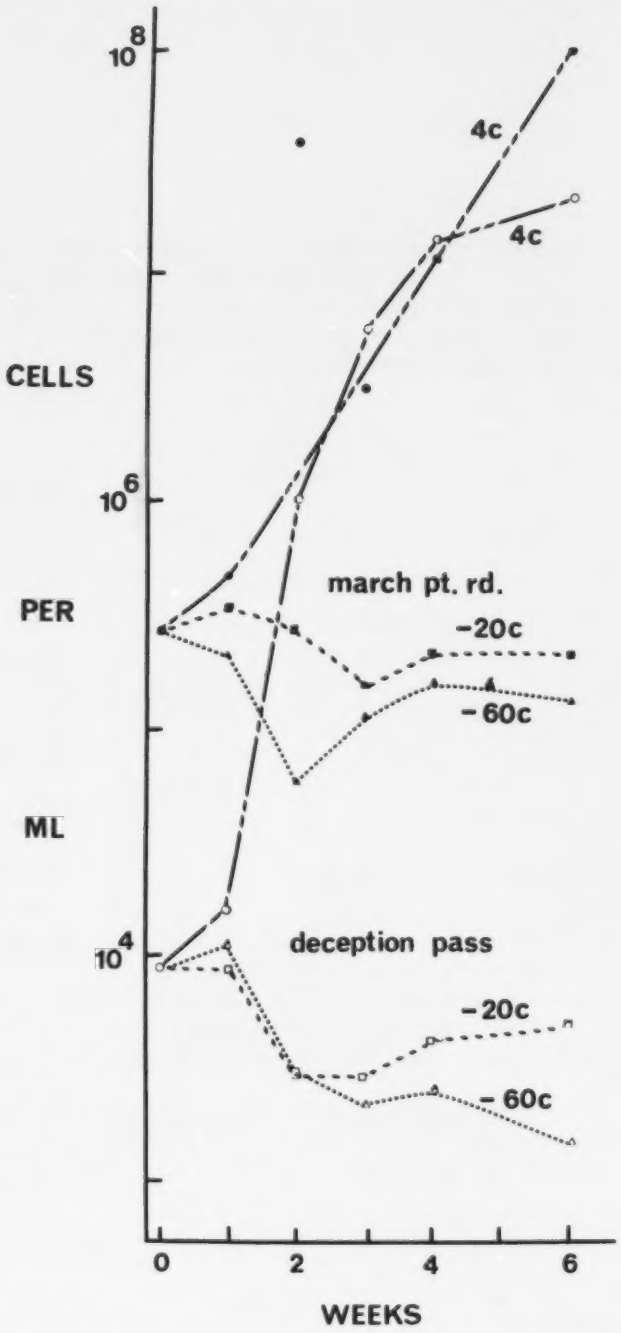

Figure 7. Effect of storage temperature on total viable numbers of bacteria in sediment (Nov., 1977) from March Pt. Rd. and West Beach (Deception Pass).

Incidence of Sulfide-generating Bacteria

The distribution of sulfide-generating bacteria in the beach and sediment samples analyzed during this survey is presented in Tables C1 and C2. The generation of sulfide from sediment populations was not affected by the addition of sulfite to the medium. The beach samples yielded more positive enrichments when sulfite was present, particularly in samples obtained during the September survey.

SECTION 6

DISCUSSION

The marine environment of this area of the Pacific northwest represents a wide diversity of conditions. Beaches range from cobble rocks to fine sands low in organic matter, to back bays where beaches and sediments are high in organic matter. Each type of environment can be found in all areas of the survey. Some sites are subject to heavy wave action, others to industial activities, while some exist in a more "pristine" situation. Consequently there was a need for a large number of sample sites in this initial survey to adequately characterize the response of the microbiological flora to oil.

The temperature of a natural environment is a critical physical parameter controlling microbial growth and activities. The microbial flora of this area will be psychrotrophic in character as the temperatures at the sample sites examined ranged from 5°C to 20°C (beach temperatures were not reported but were in general approximately five degrees warmer than reported water column values). The pH, dissolved oxygen and salinity of water column samples were characteristic of an environment which would support the growth of a typical marine psychrotrophic, heterotrophic flora.

As long as temperature is in a range which will allow microbial life, the nutritional status of an environment, in particular the nitrogen and phosphorus levels, will be the key parameter in controlling microbial heterotrophic growth if a utilizable carbon source e.g. oil is introduced to that environment. The oil-degrading activities observed in this survey confirm this: without the addition of nitrogen and phosphorus, the indigenous populaton was not able to significantly modify the n-alkane components of Prudhoe Bay oil under the experimental temperature conditions surveyed. The oil-degrading populations were more sensitive to nitrogen levels than to phosphorus levels. Increasing the temperature of incubation of nutritionally-supplemented samples resulted in a more extensive utilization of the n-alkanes and isoprenoids of Prudhoe Bay oil. Thus, factors controlling the rate of oil-utilization by bacterial populations studied in this survey are as previously reported for other marine environments (2-4).

The microbial oil-degrading populations obtained by the enrichment procedures used consisted solely of bacteria. The isolation of oil-degrading yeast and fungi would require the application of selective environmental pressures such as low pH. Such conditions would limit the growth of oil-degrading bacteria and allow the growth of oil-degrading yeast and fungi.

Differences were observed in the psychrotrophic oil-degrading capability of beach, sediment and water column samples obtained at each site as well as between sites. Differences were observed with similar materials obtained at the three different sampling times, i.e. April, September and January, used in this study. However at least one sample type from a given site always yielded bacterial populations capable of degrading n-alkanes and isoprenoids present in Prudhoe Bay oil under psychrotrophic conditions. The greatest variation in oil-degrading capacity was observed with water column samples from a high degradative index value of 3.05 in September to a low of 1.2 in January. In contrast, the oil-degrading capacity of beach samples was relatively consistent ranging from an index of 2.10 to 2.92. More samples have to be examined before the significance of these differences can be ascertained. The oil-degrading capabilities of bacterial populations obtained from sediment samples varied more than those from beach samples but less than populations from water columns. This is probably a result of the beach materials providing a more stable environment, as in soil, in contrast to the highly variable, sensitive water column environment. The sediment, as defined in this study, is considered as a transition zone between a stable beach and a sensitive water environment.

As reported in the literature (3), there was a positive relationship between oil-related industrial activities in the area, and the oil-degrading capability of the indigenous population. Consistently high values for oil-degrading activity were observed for all materials obtained from the refinery site and from E. Fidalgo Rd.

The environmental parameters observed at March Pt. Rd. and Rosario Beach sites represent two extreme conditions. The former site is near oil refineries and high in organic matter while the latter represents a "pristine" site low in organic matter. Therefore water column and sediment samples from these sites were used in "weathering" and mineraliation studies of Prudhoe Bay oil.

The weight of Prudhoe Bay oil lost due to "weathering" under laboratory conditions represents 30% of the weight of oil and is independent of sample source, at least for March Pt. Rd. and Rosario Beach materials. A further 30 to 35% by weight, can be lost due to "mineralization" by microbial activities. This gravimetric approach because of the few number of samples used in this study did not reveal any significant differences in the rate of oil utilization between March Pt. Rd. and Rosario Beach materials. The data reported were obtained with water column samples and no major problems were encountered with reproducibility of oil recovery. The values reported for weathering are similar to those noted by Atlas and Busdosh(20). However, similar experiments using sediments from these two sites, while supporting the observations reported, showed wide variation in reproducibility of replicates. This is attributed to material in the sediments interfering with the extraction of residual oil. In subsequent studies the West Beach (Deception Pass State Park) site was used in place of Rosario Beach because of the presence of more uniform particle size sand at West Beach.

The use of a more sensitive technique involving the measurement of the rate of release of $^{14}CO_2$ from ^{14}C-hexa- or ^{14}C-octadecane-"spiked" Prudhoe

Bay oil revealed a more rapid rate of release from water column samples from March Pt. Rd. than from West Beach (Deception Pass). These rates represent the total n-alkane metabolized in "spiked" Prudhoe Bay oil. A similar study using sediment samples obtained from these two sites revealed no difference between the rates of $^{14}CO_2$ release. Considerable difficulties were encountered in the initial application of this method (see Appendix B) and the results have to be considered as only being indicative of the potential of this technique. The rates reported are considerably higher than those previously observed (21-22) for n-alkanes and may be a result of the presence of larger microbial populations in the water column samples used. In addition, the use of ^{14}C-"spiked" oil versus pure hydrocarbons (21-22) could result in the differences in activities observed. As it has been noted (23) that the disappearance of n-alkanes from G.C. profiles of the saturate fraction of oil occurs prior to the period of rapid weight loss in this fraction (at least in Boreal soils) the relationships between the different rates observed with water column samples from these sites and weight loss of the different fractions of oil should beinvestigated.

Differences were noted in the response to oil of bacterial populations from West Beach (Deception Pass) and March Pt. Rd. The addition of oil to nutritionally-enriched water column samples results in an intial decrease of viability with the population from West Beach but not with that from March Pt. Rd. However both populations show a "diauxie" type of growth curve where the second growth phase of the March Pt. Rd. sample was related to n-alkane utilization. It is probable that further incubation of the West Beach (Deception Pass) sample also would have shown n-alkane utilization. Similarly a slow, more variable response of sample material from West Beach was noted in the effect of nitrogen and phosphorus levels on Prudhoe Bay oil utilization (Table 10).

The generic composition of indigenous bacterial populations present in unamended water column, sediment and beach samples consists primarily of Gram-negative rods of either the Pseudomonas or Flavobacterium genera with an occasional sample showing a high proportion of members of the Acinetobacter genus. A few samples contained members of the Gram-positive (to Gram-variable) coryneform group. Samples from two locations in the April survey, Clallam Bay and Pillar Pt., contained a relatively high content of members of this group which could be related to low salinity of the water column, due to extensive underground seepage of freshwater. Members of the Pseudomonas and Flavobacterium genera and the coryneform group also appear in varying proportions in almost all oil-degrading enrichments. The only oil-degrading population which differed was the one obtained from the sediment of False Bay, San Juan Island, which contained a high proportion of Alcaligenes spp. Member of all these groups are recognized as containing hydrocarbon-degrading species (9). The significance of the population composition and oil-degrading capability is not known. Such populations will retain their oil-degrading capability for one or two transfers on non hydrocarbon-containing medium e.g. marine agar and therefore, the possibility exists of resolving these mixed populations into axenic cultures and studying their individual roles in the degradation of oil. The temperature at which the growth and enrichments were carried out affected the composition of the population obtained, but no definite trends were apparent (9). More samples of a similar type must be examined to

determine whether trends exist as a result of temperature and sample type and/or site differences.

It is not possible to carry out a detailed comparison of the generic composition of populations from different sample types from West Beach (Deception Pass) and March Pt. Rd., because tidal conditions during the sampling period precluded the possibility of obtaining all three sample types from March Pt. Rd. Unfortunately, the dilution also was missed for the water column sample from this site which would have yielded the water column population for comparative purposes.

The differences in population composition of sediments from low organic matter-containing environments such as West Beach and organically rich environments (False Bay, San Juan Island) are reflected as well in differences in total viable population (i.e. West Beach and March Pt. Rd.). However a study of the stability of the oil-degrading capability following various sample storage conditions revealed no differences between the populations. Similar growth responses were observed although at different levels. Both populations lost a proportion of the pigmented population at 4°C but this was not reflected in changes in oil-degrading capability, as shown by the methods used in this study.

The survey of the incidence of sulfide-generating bacteria in the beach and sediment samples analyzed in this survey indicates their presence at all sites examined. The activity of these microorganisms is undoubtedly limited by the lack of suitable organic matter for growth. Such material would be provided by the products of the aerobic degradation of oil spilled in this area (Jobson, Cook, Westlake, unpublished results).

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APPENDIX A

Microbiological Media Used in this Study

(i) Artificial Seawater Solution

NaCl	23.40 g
KCl	0.75 g
$MgSO_4 \cdot 7H_2O$	7.00 g
Distilled water	1000 ml
pH	7.3

(ii) Basal Marine Agar (for viable cell counts)

proteose peptone (Difco)	1 g
yeast extract (Difco)	1 g
agar (Difco purified)	20 g
artificial sea water	1000 ml
pH adjusted to 7.5 before autoclaving	
final pH	7.3

(iii) Media for Taxonomy

April Survey

(a) Board and Holding Medium (Modified)

Solution A -

$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
yeast extract (Difco)	0.5 g
Agar (Difco purified)	5.0 g
Bromthymol blue	0.08 g
distilled water	1000 ml

Solution B -

glucose	10 g
distilled water	100 ml

Solution A and B sterilized separately, mixed, and dispensed 2 ml/ 13x100 mm test tube.

(b) Oxidase reagent - 1% solution of N,N,N',N'-tetramethyl-p-phenylenediamine-dihydrochloride

September and January Survey and other Taxonomic Studies

(a) Modified O-F Medium

Solution A -

NaCl	23.40 g
KCl	0.75 g
$MgSO_4 \cdot 7H_2O$	7.00 g
yeast extract	1.00 g

	Bromthymol blue	0.03	g
	agar (Difco-purified)	15.0	g
	distilled water	750	ml
Solution B -	glucose or lactose	10	g
	distilled water	200	ml
Solution C -	$NH_4H_2PO_4$	0.50	g
	K_2HPO_4	0.40	g
	distilled water	50	ml

The pH of all solutions was adjusted to 7.15 before autoclaving: solutions were cooled, A and B mixed, and solution C then added and the mixture dispensed into Petri plates.

(b) Oxidase reagent - as per April survey

(c) Catalase reagent - 3% H_2O_2 (freshly prepared)

(iv) Butlin's medium for sulfide-generating bacteria

(a) Butlin's

NH_4Cl	1.0	g
Na_2SO_4	2.0	g
$MgSO_4 \cdot 7H_2O$	0.02	g
Sodium lactate	1.5	ml (60% syrup)
yeast extract	1.0	g
K_2HPO_4	0.5	g
distilled water	1000	ml

Ten mls dispensed per 18x150 mm test tube and two iron finishing nails added per tube for poising the medium before autoclaving.

(b) Butlin's plus sulfite medium prepared as in (a); media sterilized, cooled and 3 drops of a freshly prepared sterile (filtration) solution of 10% Na_2SO_3 added.

(c) Saline Butlin's - prepared in artifical sea water

(v) Nitrogen and phosphorus-containing nutrient solution

10% K_2HPO_4	420 ml
10% KH_2PO_4	180 ml
NH_4NO_3	60 g

pH adjusted to 7.3 with 10N NaOH and used at the rate of 1 ml/100 ml of natural or artificial sea water medium.

APPENDIX B

Comments on the ^{14}C-radioisotope Procedure

Initial studies using phenethylamine as a CO_2-trapping agent, PPO/POPOP in toluene as a fluor, and an unrefrigerated Isocap 300 scintillation counter resulted in a large variation in the results obtained with replicate samples. The substitution of Hyamine hydroxide as the trapping agent resulted in a chemiluminescence problem which could not be overcome even by refrigeration or the addition of a few drops of glacial acetic acid or carbon tetrachloride to scintillation vials before counting. Reproducible results have been obtained using Carbo-Sorb II as a trapping agent and Monophase-40 as the fluor. Using this system any chemiluminescence can be eliminated by reprogramming the Mark I scintillation counter or using instruments programmed to make such adjustments automatically.

APPENDIX C

TABLE C1. INCIDENCE OF SULFIDE-GENERATING BACTERIA IN SEDIMENT SAMPLES

	Formation of black precipitate[a]					
	April 1977		September 1977		January 1978	
Site	$SO_4^=$	$SO_3^=/SO_4^=$	$SO_4^=$	$SO_3^=/SO_4^=$	$SO_4^=$	$SO_3^=/SO_4^=$
Birch Bay[b]	+	+	+	+	-	+
Refinery Site	+	+	+	+	+	+
Sandy Pt. (Sucia Rd.)	+	+	+	+	+	+
Samish Island (Public Beach)	+	+	+	+	+	+
March Pt. Rd.	+	+	NS	NS	+	+
E. Fidalgo Rd.	+	+	+	+	+	+
Deception Pass[b]	-	+	+	+	+	+
Pt. Partridge[b]	-	+	+	+	+	+
Ft. Worden[b]	+	+	+	+	+	+
Jamestown	NS	NS	+	+	+	+
Dungeness	+	+	+	+	+	+
Pt. Angeles	+	+	+	+	+	+
Pillar Pt.	-	+	+	+	+	+
Clallam Bay	+	+	+	+	+	+
Neah Bay	NS	NS	+	+	NS	NS
Lopez Island						
Shoal Bight	NS	NS	+	+	NS	NS
San Juan Island						
South Beach	NS	NS	+	+	NS	NS
False Bay	NS	NS	+	+	NS	NS
Orcas Island						
Crescent Beach	NS	NS	+	+	NS	NS
Terrill Beach	NS	NS	+	+	NS	NS

[a]+=formation of black precipitate; -=no precipitate formed after 3 weeks incubation at room temperature; April samples - normal Butlin's medium; September and January samples - saline Butlin's (See Appendix A).
[b]State Park
NS=not sampled

TABLE C2. INCIDENCE OF SULFIDE-GENERATING BACTERIA IN BEACH SAMPLES

Site	Formation of black precipitate[a]					
	April 1977		September 1977		January 1978	
	$SO_4^=$	$SO_3^=/SO_4^=$	$SO_4^=$	$SO_3^=/SO_4^=$	$SO_4^=$	$SO_3^=/SO_4^=$
Birch Bay[b]	+	+	-	+	+	+
Refinery Site	+	+	-	+	+	+
Sandy Pt. (Sucia Rd.)	+	+	-	+	-	+
Samish Island (Public Beach)	+	+	-	+	-	+
March Pt. Rd.	+	+	-	+	+	+
E. Fidalgo Rd.	+	+	-	+	-	+
Deception Pass[b]	-	+	-	+	-	-
Pt. Partridge[b]	-	+	+	+	+	+
Ft. Worden[b]	+	+	-	+	-	+
Jamestown	NS	NS	-	+	+	+
Dungeness	-	+	+	+	+	+
Pt. Angeles	+	+	-	+	-	+
Pillar Pt.	-	+	-	+	NS	NS
Clallam Bay	-	+	-	+	-	-
Neah Bay	NS	NS	-	-	+	+
Lopez Island Shoal Bight	NS	NS	-	+	NS	NS
San Juan Island South Beach	NS	NS	-	+	NS	NS
False Bay	NS	NS	+	+	NS	NS
Orcas Island Crescent Beach	NS	NS	+	+	NS	NS
Terrill Beach	NS	NS	+	+	NS	NS

[a]+=formation of black precipitate; -=no precipitate formed after 3 weeks incubation at room temperature; April samples - normal Butlin's medium; September and January samples - saline Butlin's (see Appendix A)
[b]State Park
NS=not sampled

APPENDIX D

TABLE D1. pH WATER COLUMN SAMPLES

Site	pH		
	April '77	Sept. '77	Jan. '78
Birch Bay[a]	8.1	7.9	7.7
Refinery Site	8.0	7.7	7.7
Sandy Point (Sucia Road)	7.7	7.6	6.9
Samish Island (Public Beach)	7.7	7.9	7.7
March Pt. Road	7.7	7.5	6.9
E. Fidalgo Road	8.3	7.4	7.7
Deception Pass[a]	7.8	7.8	8.0
Pt. Partridge[a]	7.9	7.8	7.6
Ft. Worden[a]	8.2	8.1	7.6
Jamestown	-[b]	8.2	7.1
Dungeness	8.1	8.1	7.5
Pt. Angeles	8.2	8.0	6.6
Salt Creek[a]	8.9	-	-
Pillar Point	8.3	7.8	7.8
Clallam Bay	7.5	7.8	8.1
Neah Bay	-	8.0	6.8
Lopez Island[c]			
Shoal Bight	-	8.1	-
San Juan Island[c]			
South Beach	-	7.7	-
False Bay	-	7.3	-
Orcas Island[c]			
Crescent Beach	-	7.9	-
Terrill Beach	-	7.9	-

[a]State Park
[b]not sampled
[c]only sampled in September, 1977

TABLE D2. TEMPERATURE - WATER COLUMN SAMPLES

Site	Temperature (°C) April '77	Sept. '77	Jan. '78
Birch Bay[a]	9.9	16.0	6.2
Refinery Site	9.3	16.0	4.5
Sandy Point (Sucia Road)	8.3	14.0	5.5
Samish Island (Public Beach)	12.6	12.0	6.2
March Pt. Rd.	12.6	12.5	6.3
E. Fidalgo Rd.	8.3	13.5	10.5
Deception Pass[a]	10.5	11.0	11.5
Pt. Partridge[a]	10.5	11.0	7.0
Ft. Worden[a]	12.0	12.0	9.8
Jamestown	_[b]	14.0	9.3
Dungeness	12.0	16.0	7.5
Pt. Angeles	9.7	11.0	8.3
Salt Creek	9.0	-	-
Pillar Pt.	12.0	11.2	8.4
Clallam Bay	9.0	11.0	9.3
Neah Bay	-	12.0	9.0
Lopez Island[c]			
Shoal Bight	-	12.8	-
San Juan Island[c]			
South Beach	-	11.0	-
False Bay	-	10.0	-
Orcas Island[c]	-	10.0	-
Crescent Beach	-	15.0	-
Terrill Beach	-	12.0	-

[a]State Park
[b]not sampled
[c]only sampled in September, 1977

TABLE D3. DISSOLVED OXYGEN CONTENT - WATER COLUMN SAMPLES

Site	Do (ppm) April '77	Sept. '77	Jan. '78
Birch Bay[a]	9.3	8.6	10.7
Refinery Site	-[b]	8.7	10.3
Sandy Point (Sucia Road)	-	8.2	10.6
Samish Island (Public Beach)	19.6	7.4	10.4
March Pt. Rd.	13.0	6.1	11.0
E. Fidalgo Rd.	13.5	5.3	10.4
Deception Pass[a]	9.0	6.9	10.6
Pt. Partridge[a]	9.1	7.6	10.4
Ft. Worden[a]	13.6	8.2	10.4
Jamestown	-	11.1	10.1
Dungeness	9.7	8.9	10.0
Pt. Angeles	10.6	9.2	9.3
Salt Creek[a]	8.9	-	-
Pillar Pt.	10.8	7.2	11.1
Clallam Bay	10.2	7.8	11.8
Neah Bay	-	8.0	10.4
Lopez Island[c]			
Shoal Bight	-	10.5	-
San Juan Island[c]			
South Beach	-	8.5	-
False Bay	-	6.2	-
Orcas Island[c]			
Crescent Beach	-	8.8	-
Terrill Beach	-	8.8	-

[a]State Park
[b]not sampled
[c]only sampled in September, 1977

TABLE D4. SALINITY - WATER COLUMN SAMPLES

Site	Salinity (ppt)		
	April '77	Sept. '77	Jan. '78
Birch Bay[a]	28.6	26.5	19.7
Refinery Site	29.3	26.0	20.6
Sandy Point (Sucia Road)	28.0	27.5	20.8
Samish Island (Public Beach)	28.7	27.4	19.8
March Pt. Rd.	28.7	27.2	16.5
E. Fidalgo Rd.	28.5	27.7	19.5
Deception Pass[a]	28.8	30.0	20.8
Pt. Partridge[a]	29.8	29.4	21.8
Ft. Worden[a]	29.5	29.7	22.2
Jamestown	-[b]	29.8	22.0
Dungeness	29.5	28.5	20.3
Pt. Angeles	28.6	30.9	18.8
Salt Creek[a]	30.7	-	-
Pillar Pt.	20.4	29.8	18.3
Clallam Bay	20.9	30.1	21.9
Neah Bay	-	30.0	20.8
Lopez Island[c]			
Shoal Bight	-	27.5	-
San Juan Island[c]			
South Beach	-	28.8	-
False Bay	-	29.4	-
Orcas Island[c]			
Crescent Beach	-	27.4	-
Terrill Beach	-	28.1	-

[a]State Park
[b]not sampled
[c]only sampled in September, 1977

TABLE D5. G.C. PROFILE OF PENTANE EXTRACT OF RECOVERED PRUDHOE BAY OIL AFTER 28 DAYS INCUBATION AT 8°C WITH WATER COLUMN SAMPLES

	G.C. Profile[a]		
Site	April '77	Sept. '77	Jan. '78
Birch Bay[b]	N	N	N
Refinery Site	N	N	N
Sandy Point (Sucia Road)	N	N	N
Samish Island (Public Beach)	N	N	N
March Pt. Rd.	N	N	N
E. Fidalgo Rd.	N	N	N
Deception Pass[b]	N	N	N
Pt. Partridge[b]	N	N	N
Ft. Worden[b]	N	N	N
Jamestown	-[d]	N	N
Dungeness	N	N	N
Pt. Angeles	N	N	N
Salt Creek[b]	N	-	-
Pillar Pt.	N	N	N
Clallam Bay	N	N	N
Neah Bay	-	S	N
Lopez Island[c]			
Shoal Bight	-	N	-
San Juan Island[c]			
South Beach	-	N	-
False Bay	-	N	-
Orcas Island[c]			
Crescent Beach	-	N	-
Terrill Beach	-	N	-

[a] N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}
[b] State Park
[c] only sampled in September, 1977
[d] not sampled

TABLE D6. G.C. PROFILE OF PENTANE EXTRACT OF RECOVERED PRUDHOE BAY OIL AFTER 28 DAYS INCUBATION AT 8°C WITH SEDIMENT SAMPLES

	G.C. Profile[a]		
Site	April '77	Sept. '77	Jan. '78
Birch Bay[b]	N	N	N
Refinery Site	N	S	-
Sandy Point (Sucia Road)	N	N	N
Samish Island (Public Beach)	N	N	N
March Pt. Rd.	N	N	N
E. Fidalgo Rd.	P	N	N
Deception Pass[b]	N	N	N
Pt. Partridge[b]	N	N	N
Ft. Worden[b]	N	N	N
Jamestown	-[d]	N	N
Dungeness	N	N	-
Pt. Angeles	N	N	N
Salt Creek[b]	-	-	-
Pillar Pt.	N	N	-
Clallam Bay	N	N	N
Neah Bay	-	N	N
Lopez Island[c]			
Shoal Bight	-	N	-
San Juan Island[c]			
South Beach	-	N	-
False Bay	-	P	-
Orcas Island[c]			
Crescent Beach	-	N	-
Terrill Beach	-	N	-

[a]N=no degradation; S=selective metabolism, C_{12} to C_{19}; P=partial removal n-alkane peaks still discernible
[b]State Park
[c]only sampled in September, 1977
[d]not sampled

TABLE D7. G.C. PROFILE OF PENTANE EXTRACT OF RECOVERED PRUDHOE BAY OIL AFTER 28 DAYS INCUBATION AT 8°C WITH BEACH SAMPLE

Site	G.C. Profile[a]		
	April '77	Sept. '77	Jan. '78
Birch Bay[b]	N	-	N
Refinery Site	N	-	N
Sandy Point (Sucia Road)	N	N	N
Samish Island (Public Beach)	N	N	N
March Pt. Rd.	N	S	-
E. Fidalgo Rd.	N	N	N
Deception Pass[b]	N	N	N
Pt. Partridge[b]	N	N	N
Ft. Worden[b]	N	N	N
Jamestown	-[d]	S	N
Dungeness	N	N	N
Pt. Angeles	N	N	N
Salt Creek[b]	-	-	-
Pillar Pt.	N	N	-
Clallam Bay	N	N	N
Neah Bay	-	N	N
Lopez Island[c]			
Shoal Bight	-	N	-
San Juan Island[c]			
South Beach	-	N	-
False Bay	-	N	-
Orcas Island[c]			
Crescent Beach	-	S	-
Terrill Beach	-	N	-

[a]N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}
[b]State Park
[c]only sampled in September, 1977
[d]no sample

TABLE D8. EFFECT OF TEMPERATURE, INCUBATION TIME AND SAMPLE TYPE OBTAINED IN JULY, 1977 ON THE G.C. PROFILE OF PRUDHOE BAY OIL RECOVERED AFTER INCUBATION AT 8 OR 20°C

		March Pt. Road				Rosario			
		Water Column		Sediment		Water Column		Sediment	
	Time (days)	G.C. Profile	Total bacterial count x10^6/ml[b]	G.C. Profile	Total bacterial count x10^6/ml	G.C. Profile	Total bacterial count x10^6/ml	G.C. Profile	Total bacterial count x10^6/ml
8°C	7	N	0.7 ± 0.05	N	13.2 ± 6.9	N	3.3 ± 0.3	N	3.6 ± 0.4
	14	I	31 ± 4.6	S	21.0 ± 8.1	S	48 ± 5.4	N	3.9 ± 0.2
	21	C	272 ± 86.6	S	32 ± 7.5	C	440 ± 103	N	22.1 ± 3.0
	28	C	266 ± 31.9	I	47 ± 8.2	C	408 ± 31.8	P	116 ± 12.5
	35	C	364 ± 52.4	I	55 ± 7.7	C	558 ± 35.4	P	87 ± 17.8
	42	C	450 ± 64	C	21.3 ± 2.1	C	315 ± 28.3	I	87 ± 8.4
	49	C	550 ± 72	-	-	C	310 ± 23.6	I	137 ± 14.3
	56	C	242 ± 17.4	C	19.3 ± 1.1	C	255 ± 35.3	I	110 ± 10.2
20°C	5	I	133 ± 17.4	I	287 ± 14.0	I	466 ± 39.1	N	12.5 ± 0.5
	8	I	242 ± 30.9	I	235 ± 23.5	C	470 ± 64	C	290 ± 26.5
	13	C	229 ± 27.0	C	120 ± 17.8	C	301 ± 19.1	C	320 ± 48
	20	C	236 ± 61.5	C	18.7 ± 17.1	C	354 ± 62.8	C	400 ± 89
	48	C	4.8 ± 6.9	C	13.5 ± 2.14	C	185 ± 24.0	C	73 ± 8.5

[a]N=no degradation; S=selective metabolism, n-alkanes C_{12} to C_{19}; P=partial removal, n-alkane peaks still discernible; I=only isoprenoid peaks remaining; C=complete utilization of n-alkanes and isoprenoids

[b]average ± one standard deviation

TABLE D9. AVAILABLE PHOSPHORUS CONTENT OF BEACH, SEDIMENT AND WATER COLUMN SAMPLES

		µg P/g[a]		µg P/ml[a]
Sampling	Site	Beach	Sediment	Water
July '77	March Pt. Rd.	-	14.0[b] (13.7)[c]	0.67
	Rosario Beach	-	2.6 (47)	0.10
Sept. '77	March Pt. Rd.	5.75 (6.38)	-	0.16
	Deception Pass	2.25 (5.38)	1.46 (3.63)	0.04
	Clallam Bay	1.46 (3.38)	2.95 (4.25)	0.04
	Jamestown	3.44 (8.75)	4.79 (5.38)	0.07
	Crescent Beach (Orcas Island)	2.51 (4.00)	2.74 (6.25)	0.03
	False Bay (San Juan Island)	4.49 (8.25)	28.0 (36.88)	-

[a]ascorbic acid-ammonium molybdate method
[b]extracting solution - 0.5M $NaHCO_3$
[c]extracting solution - 0.03N NH_4F + 0.03NH_2SO_4

TABLE D10. SUSPENDED SOLIDS CONTENT OF REPRESENTATIVE WATER COLUMN SAMPLES

Site[a]	Suspended solids mg/l
March Pt. Rd.	35.8
Deception Pass	6.6
Clallam Bay	6.3
Jamestown	35.1
Crescent Beach	12.7

[a]Sampled September, 1977

TABLE D11. GENERIC COMPOSITION OF INDIGENOUS BACTERIAL POPULATIONS OF BEACH SAMPLE (APRIL, 1977)

Site	% Generic Composition						
	Alcaligenes	coryneforms	Cytophaga	Flavobacterium	Pseudomonas	Vibrio	Unidentified
Birch Bay[a]	38.5	-[b]	-	12.2	37.0	-	12.6
Refinery Site	18.5	18.9	-	10.9	-	-	51.8
Sandy Pt. (Sucia Rd.)	17.4	-	-	-	70.4	-	12.1
Samish Island (Public Beach)	8.9	36.5	-	-	5.8	9.2	39.6
March Pt. Rd.	8.5	-	-	16.9	13.2	-	63.3
E. Fidalgo	2.7	-	-	65.6	19.0	11.8	1.0
Deception Pass[a]	-	-	-	-	100	-	-
Pt. Partridge[a]	15.4	-	-	15.5	34.1	-	35.4
Dungeness	0.6	3.1	-	75.2	17.8	-	3.1
Clallam Bay	-	83.3	-	8.3	6.7	-	1.7
Pillar Pt.	42.6	49.2	-	-	1.8	-	6.2
Pt. Angeles	3.9	-	1.6	22.9	50.6	-	20.8
Ft. Worden[a]	21.7	-	-	7.0	12.0	-	59.1

[a]State Park
[b]no colonies present on dilution plate used to assess population composition

TABLE D12. GENERIC COMPOSITION OF INDIGENOUS BACTERIAL POPULATIONS OF SEDIMENT SAMPLES (APRIL, 1977)

Site	% Generic Composition						
	Alcaligenes	coryneforms	*Cytophaga*	*Flavobacterium*	*Pseudomonas*	*Vibrio*	Unidentified
Birch Bay[a]	-[b]	-	-	10.5	14.2	4.8	70.4
Refinery Site	18.3	29.7	-	6.3	-	18.6	27.1
Sandy Pt. (Sucia Rd.)	7.2	-	-	6.7	45.8	-	40.5
Samish Island (Public Beach)	-	36.8	-	0.7	10.3	4.7	39.5
March Pt. Rd.	-	10.6	-	6.0	42.0	11.2	25.2
E. Fidalgo Rd.	2.1	2.9	-	57.5	22.2	11.8	3.4
Deception Pass[a]	19.6	-	1.4	4.6	58.1	-	16.3
Pt. Partridge[a]	23.0	-	9.6	5.1	62.3	-	-
Dungeness	-	-	-	52.7	8.2	-	38.9
Clallam Bay	2.2	1.8	6.3	34.3	16.9	4.9	33.8
Pillar Pt.	6.9	-	-	5.0	6.0	-	82.1
Pt. Angeles	18.4	-	5.8	20.5	17.3	3.7	34.2
Ft. Worden[a]	12.6	-	15.4	1.4	46.7	3.7	20.1

[a]State Park
[b]no colonies present on dilution plate used to assess population composition

TABLE D13. GENERIC COMPOSITION OF MARCH PT. RD. WATER COLUMN POPULATIONS (SEPT., 1977) AFTER ENRICHMENT IN THE PRESENCE OF NITROGEN, PHOSPHORUS AND PRUDHOE BAY OIL AT 8°C AND 20°C

	% Generic Composition	
Genus	8°	20°C
Alcaligenes sp.	-	7.3 (2)[b]
coryneforms	5.7 (2)	0.7 (1)
Flavobacterium sp.	60.2 (2)	45.5 (3)
Pseudomonas sp.	27.8 (3)	39.8 (6)
Unidentified[c]	2.8 (3)	6.8 (1)
Non-transferable colonies[d]	3.4 (1)	-

[a]no colonies present on dilution plate used to assess population composition
[b]number of colonial types in parentheses
[c]similar to either *Alcaligenes* or *Pseudomonas* spp. with the exception that isolates were capable of non acid-generating anaerobic growth
[d]colonies would not grow on transfer